MOLECULAR CHARACTERIZATION OF *SULFOBACILLUS* AND RELATED ORGANISMS

BY

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

Thirteen *Sulfobacillus* strains from different geographical locations and two *Alicyclobacillus* strains were included in this study. These organisms proved to be moderately thermophillic (two different sets of optimal temperatures of 45°C and 55°C were found), Gram-positive, endospore forming bacteria. The pH optima of the strains tested was pH 2.5 and the pH range lay between pH 1.5 and pH 5.0. It was established that some strains of *Sulfobacillus* had the capacity for anaerobic growth when using ferric iron as an electron donor. It was determined that *S. thermosulfidooxidans* was the species found within South African biooxidation plants. Plasmids were identified within strain 611 (*S. thermosulfidooxidans*) isolated from a Billiton commercial plant.

The sample of *Sulfobacillus* strains used in this study could clearly be divided into two groups based on the analysis of their 16S rRNA gene sequences as well as the number of ribosomal (*rrn*) operons present as determined by Southern hybridization.

A system for the convenient identification of *Sulfobacillus* species was developed using several of the techniques employed in this study. Preliminary identifications can be obtained by restriction enzyme digestion of the PCR amplified 16S rRNA gene. Confirmation of this placement can be done by comparison of the 16S – 23S rRNA spacer region amplification band sizes. Once the preliminary identification has been completed it is possible to place the isolate in the correct species by making use of the differences in sugar utilization that the species exhibit. The more laborious method of 16S rRNA sequence comparisons can be undertaken if there is still any uncertainty as to which species an isolate belongs to.

Phylogenetic results obtained from the 16S rRNA gene sequence indicates that the genus *Sulfobacillus* should probably be divided into two individual genera. Further information gathered from the phylogenetic comparisons indicates that strain Riv-14 previously assigned to *S. ambivalens* is more closely related to *S. montseratensis*. Data obtained from 16S – 23S rRNA spacer region analysis confirms this result.

Future work includes the use of DNA-DNA hybridization studies and mol% G+C ratio's in order verify the presence of two distinct genera as well as placing Riv-14 within the correct species.

OPSOMMING

Dertien isolate van die genus *Sulfobacillus* afkomstig van geografies verskillende areas en twee isolate van die genus *Alicyclobacillus* is in die studie ingesluit. Hierdie organismes het gewys dat hulle gematigde termofiele (twee verskillende groepe met optimale temperature van 45°C en 50°C elk was waargeneem), Gram-positiewe, endospoorvormende organismes is. Die pH optima van die isolate was pH 2.5 en die reeks van pH waar groei moontlik was het tussen pH 1.5 en pH 5.0 gelê. Dit was bewys dat sekere van die *Sulfobacillus* isolate oor die vermoë beskik het om anaerobies te respireer deur ferri yster (Fe^{3+}) as elektron akseptor te gebuik. Dit was bepaal dat *S. thermosulfidooxidans* die spesies is wat teenwoordig was in die bio-oksidasie reaktors in Suid Afrika. Plasmiede vanuit die isolaat 611 (*S. thermosulfidooxidans*) afkomstig vanuit 'n Billiton komersieële reaktor, is geidentifiseer.

Die toetsmonster van *Sulfobacillus* isolate gebruik in hierdie studie het duidelik daarop gewys dat daar twee groepe binne *Sulfobacillus* is. Hierdie stelling is gebaseer op data afkomstig van die analiese van die 16S rRNA volgorde asook die aantal ribosomale (*rrn*) kopieë teenwoordig soos bepaal deur Southern klad eksperimente.

'n Sisteem vir die maklike identifikasie van *Sulfobacillus* spesies is ontwerp deur van verskeie tegnieke, soos in hierdie studie toegepas, gebruik te maak. Aanvanklike identifikasie kan verkry word deur gebruik te maak van restriksie ensiem vertering van PKR geamplifiseerde 16S rRNA geen. Hierdie plasing van die isolaat kan bevestig word deur die grootte van die 16S – 23S rRNA intergeniese amplifikasie produkte te vergelyk. Sodra die aanvanklike plasing van die isolaat voltrek is, kan daar van die verskille in die vermoëns van die spesies om sekere suikers the benut, gebruik gemaak word om die isolaat binne die regte spesies te plaas. Die meer werksintensiewe metode van 16S rRNA volgorde vergelyking kan gebruik word indien daar enige onsekerheid is oor by watter spesies die isolaat hoort.

Filogenetiese resultate verkry van die vergelyking van die 16S rRNA geen volgorde dui daarop aan dat die genus *Sulfobacillus* waarskynlik uit meer as een genus bestaan. Die filogenetiese data dui verder daarop dat die isolaat Riv-14 wat as 'n *S. ambivalens* geklassifiseer is, nader verwant is aan die spesies *S. montseratensis*. Data verkry vanaf die 16S – 23S intergeniese gebied analiese bevestig hierdie resultaat.

Toekomstige werk sluit DNA-DNA hibridisasie en mol% G+C ratio eksperimente in om sodoende die teenwoordigheid van meer as een genus sowel as die plasing van Riv-14 in die korrekte spesies te bevestig.

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ABBREVIATIONS

A	adenosine
ala	alanine
ATP	adenosine 5'-triphosphate
bp	base pairs
C	cytosine
CHEF	contour clamped homogenous electric field
CsCl	cesium chloride
Cu^{2+}	copper ion
$CuFeS_2$	chalcopyrite
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycitosine 5'-triphosphate
dGTP	deoxyguanine 5'-triphosphate
DIG	dioxygeni-11-dUTP
DNA	deoxyribonucleic acid
dTTP	deoxythymine 5'-triphosphate
dUTP	deoxyuradine 5'-triphosphate
EDTA	ethylene-diaminetetra-acetic acid
EPS	exopolysaccharide/extracellular polymeric substance
ESC	evolutionary species concept
FeS_2	pyrite
g	gram
µg	microgram(s)

G	guanine
g/t	gram/ton
G+C	guanine:cytosine ration
gDNA	genomic DNA
glu	glutamine
ile	isoleucine
IR	intergenic region
kb	kilo bases
l	litre
LFRFA	low frequency restriction fragment analysis
M	molar
mg	milligram(s)
ml	millilitre(s)
mm	millimeter(s)
mM	millimolar
μM	micro molar
mol%	molar persentage
MoS_2	molebdenite
MS	metal sulphides
NCBI	National Center for Biotechnology Information
ng	nanogram(s)
nm	nanometer(s)
OD	optical density
OD600nm	optical density at 600 nanometers

PbS	galena
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
PhSC	polythetic species concept
PSC	polygenetic species concept
PTT	potassium tetrathionate
RAPD	random amplified polymorphic DNA
RBR	relative binding ratio
rDNA	ribosomal deoxyribonucleic acid
rpm	revolutions per minute
rrn	ribosomal RNA
s	second(s)
S^{2-}	sulphide ion
SDS	sodium dodecyl sulphate
SE/EW	solvent extraction and electorwinning
spp	species
SSC	saline-sodium citrate
T	thymine
TBE	tris-borate EDTA buffer
TE	tris EDTA buffer
T_M	melting temperature
ΔT_M	change in melting temperature
tpm	tons per months
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer deoxyribonucleic acid
U	unit of enzyme activity
UV	ultraviolet

V	volts
v/v	volume per volume
w/v	weight per volume
WS_2	tungstenite
YE	yeast extract
ZnS	spalerite
α	alpha
Δ	delta
Λ	lambda
μ	micro
ω	omega

CHAPTER ONE

LITERATURE REVIEW

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1.1 Biomining introduction

Biomining is a general term that incorporates the principles of bioleaching and biooxidation. When a bacterium is used to enhance the extraction of a metal without rendering the metal soluble, the term biooxidation is applied. Bioleaching is the process in which an insoluble metal is converted to a soluble form and transferred into a liquid. Both processes rely on oxidation and can thus be classified as biooxidation processes, but only when a metal is leached into solution can the term bioleaching be applied.

The extraction of metal from ores by the oxidizing abilities of microbes is an ancient, naturally occurring phenomenon. Biomining has been practiced throughout history even though the early miners were unaware of the contribution from the microbial communities. One of the earliest documented reports of the use of biomining is that of the copper mining activities of the Rio Tinto mine in Spain during the 18th century.

In recent years the need to find an alternative to conventional mining operations has increased. The major reason for this increase is the rapid depletion of higher-grade ores. Many of the lower grade ores are not an economically viable option for metal recovery by means of conventional mining methods, but by utilizing microbes for metal extractions some of these non-profitable ores can now be economically recovered. Another advantage of biomining over conventional mining methods is that biomining usually has a less harmful effect on the environment. The classical mining techniques rely on smelting or roasting of the ores, which results in the release of harmful gaseous emissions such as sulfur dioxide. In addition these physicochemical processes require enormous amounts of energy, whereas the more environmentally friendly biomining has a comparatively low energy need. Mine tailings and waste products produced by physicochemical mining techniques are known to be highly reactive and may be biologically leached, resulting in unwanted acid and metal pollution of the water systems. Tailings and other wastes from biomining processes support reduced microbial activity mostly because of the extent to which they have already been leached. Another environmental benefit of biomining, specifically the bioleaching process, is the ability to remove sulfur from coal reserves. This also decreases the amount of harmful sulfur dioxide released during the burning of these fossil fuels (Rawlings, 1995, Ehrlich, 1990).

There are two types of processes employed that make use of microorganisms for mining purposes namely irrigation type and stirred tank type systems. One of the features that these two processes have in common is that neither is conducted under sterile conditions. In most commercial fermentation processes, sterility is of the utmost importance, but within the biomining industry, a sterile setting is not required as the environment in which the biomining organisms flourish is inhospitable to most other organisms.

1.2 Irrigation type systems

Irrigation type systems are very simple systems where the crushed ore is stacked in columns, dumps or heaps and then subjected to chemical and biological degradation. Several cases have also been reported where irrigation of an ore body was done *in situ* (Schnell, 1997; Brierley 1997: Brierley and Brierley 1999). Copper is mostly recovered by this means. Previously irrigation type systems were used for the *in situ* mining of uranium but these operations have been abandoned (McCready 1988, McCready and Gould, 1989).

1.2.1 Dump leaching

Dump leaching was initiated during the late 1960's and is the most rudimentary and crudest of all the biomining operations. The uncrushed ore is piled high to form large dumps. An acidic leaching solution is sprayed on top of the dumps and allowed to percolate slowly through the ore. Solution spraying allows the incorporation of oxygen, which is essential to both chemical and biological oxidation. With dump leaching, the leaching solution is not inoculated with bacteria, but relies on the natural proliferation of these ubiquitously occurring bacteria under suitable conditions. The metal-rich or "pregnant" solution is collected from the bottom of the dump and the solubilised metal extracted. Cementation, or solvent extraction and electrowinning (SX/EW), is normally used to extract solubilised copper (Cu^{2+}) from the leaching solution. Cementation is the process whereby ferrous iron is used to replace the copper in solution (Brieley C.L., 1982, Schnell 1997). SX/EW is made up of three closed loops in which the "pregnant" leaching solution is first brought into contact with an organic mixture (Cu^{2+} extracted into the organic solution), then a recycled electrolyte strips the Cu^{2+} from the organic solution and finally it is transported to the electrowinning plant. At the electrowinning plant the highly purified copper is recovered by electrodeposition. The now "barren" solution is recycled to the top of the dump and reused as the leaching solution. Dump leaching normally takes place over several years in order to extract as much of the metal as possible. One of the main reasons why this process is so lengthy is the large size of the rocks within the body of ore and the inefficiencies in irrigation and aeration.

1.2.2 Heap leaching

Heap leaching is very similar to dump leaching with the biggest difference between the two being their efficiencies. Heap leaching is made more effective by crushing the ore and agglomerating it with acid so that finer particles are bound with larger particles (Schnell,1997). The agglomerated ore is stacked on impermeable irrigation pads to minimize the loss of "pregnant" solution at the end of the oxidation cycle. The biooxidation process is speeded up tremendously by the inclusion of aeration pipes. This ensures a constant supply of oxygen to areas deep within the heap where oxidation processes are occurring. Specific inorganic nutrients such as $(NH_4)_2SO_4$ and KH_2PO_4 are frequently added to the leaching solution prior to irrigation of the heap. Inoculation of heaps may speed up the leaching rate, but even distribution of microbes is difficult to achieve and the process frequently relies on natural dispersion of microbes within the heap. Nevertheless, heap leaching is more efficient than dump leaching and usually ores of a higher grade are treated within these processes. The increased efficiency of heap leaching results in the completion of the biooxidation processes in months rather than years.

The metal chiefly recovered by this process is copper. Recently, refractory gold-bearing ores have also been treated using heap leaching. The ore is agglomerated in the same way as copper-containing ore and stacked in the heap. The irrigation solution used in bioleaching of refractory gold is similar to that used with copper-containing ore except that the bioleaching solution is supplemented with ferric iron and bacteria when first used to irrigate the heap iron (Brierley J.A., 1997). This is a bioleaching process, which implies that the gold remains within the heap once the biooxidation process is complete. After the heap has been washed and treated to remove excess acid and cyanide-consuming compounds, the ore is agglomerated with lime and restacked. A dilute solution of cyanide is used to extract the gold from the heap. This process allows the extraction of gold from very low-grade ore (as low as 1g Au per ton), which would otherwise not have been considered an economically viable option (Brierley J.A., 1997).

1.2.3 *In situ* leaching

In situ leaching is used to extract metals directly from the site of excavation. An acidified leaching solution is percolated through the roof or walls of the site and collected in centrally placed production wells. From here the "pregnant" solution is pumped to the surface where the metal is extracted. This process is normally used for the recovery of metals from lower-grade ores in inaccessible sites and abandoned mine workings (Brierley C.L., 1982). It has often been found that *in situ* leaching has a very low impact on the environment and the only prerequisite for *in situ* mining is a suitable geology. This process has been successfully used in the recovery of both copper and uranium (McCready, 1988, McCready and Gould, 1989, Schnell, 1997), although the *in situ* leaching of uranium no longer takes place (Rawlings, 2002).

1.3 Stirred tank processes

These processes function at a higher rate and are more efficient than irrigation type systems. Stirred tank reactors are very expensive to construct and to operate and, therefore, only high value ore is used within these systems. One of the reasons why these systems are quite expensive to operate is the extensive cooling systems that are required to maintain a constant temperature. Biooxidation is an exothermic process and the excess heat that is produced has to be removed from the reactors in order to maintain a temperature at which the biomining organisms can survive. A typical arrangement of these bioreactors can be seen in Figure.1.1. Normally the bioreactors are arranged in series and operated in a continuous flow mode. During the first stages of biooxidation the tanks are placed in parallel so that an adequate retention time can be achieved to prevent cell wash-out and to allow the microbial cell numbers to reach steady-state levels.

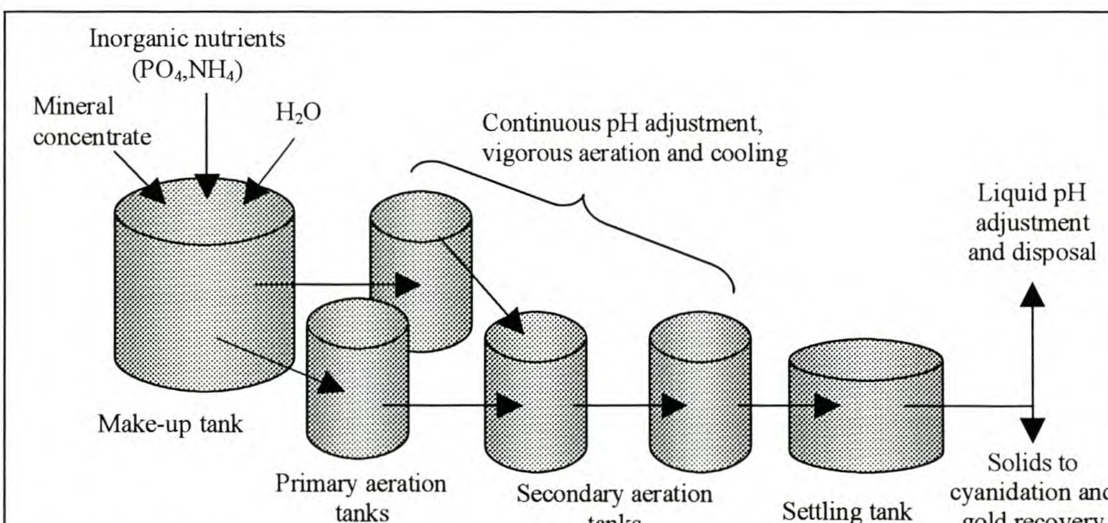

Figure 1.1. Flow diagram (adapted from Rawlings 2002) depicting a typical continuous flow biooxidation plant for the pre-treatment of refractory gold ore. Placing several primary aeration tanks in parallel increases the retention time above that of the cell doubling time, ensuring that microbial cell wash-out does not occur during mineral treatment.

The microbial communities within stirred tank bioreactors that treat gold bearing ores have been studied in most detail. Within tanks operating at 40°C, *Leptospirillum* and *Acidithiobacillus caldus* were found to dominate (Rawlings, 1995). *Acidithiobacillus* *ferrooxidans* was either not detectable or present in low numbers. There is very limited information pertaining to bacterial communities found within reactors operating at temperatures around 50°C. Fatty acid composition studies were used in an attempt to shed some light on this issue. Organisms with a fatty acid profile similar, but not identical, to *Acidithiobacillus* were found to dominate, while a significant number of the species *Sulfobacillus thermosulfidooxidans* was also present (Franzmann and Williams, 1997).

1.3.1 BIOX® process

Stirred tank technologies are mostly used for the pre-treatment of recalcitrant gold bearing ores by the BIOX®-process. Recalcitrant ores are those in which the gold is encased within a mixture of pyrite/arsenopyrite. This combination is not easily solubilised by conventional cyanidation processes and requires pre-treatment in order to decompose the pyrite/arsenopyrite (Rawlings, 2002). Once the pyrite and arsenopyrite is decomposed, the gold can be extracted using cyanide.

The first commercial operation using biooxidation of gold bearing ores was implemented in 1986 at the Fairview mine in Barberton, South Africa. The BIOX® process has several advantages over the conventional gold mining methods such as roasting, pressure oxidation and nitric acid leaching. Some of these include improved rates of gold recovery at a significantly lower cost, low running costs with very little skills required for the process operation and an ongoing process development and improvement. The BIOX® plant at the Fairview mine is a notable success story. Table 1.1 gives a summary of the performance of this plant from 1988 up to 1999. Figure 1.2 is a schematic representation of a typical BIOX® process.

Table 1.1 Summary of the Fairview BIOX® plant performance from 1988-1998 (www.goldfields.co.za/profile/technology)

	Yearly average						
	1988	**1990**	**1991**	**1995**	**1996**	**1997**	**1998**
Concentrate treated (tpm)	263	350	712	906	754	865	901
Concentrate gold grade (g/t)	99	109	127	151	127	116	118
Concentrate S^{2-} grade (%)	27.4	23.1	22.9	18.0	16.8	14.3	14.2
Gold recovery in cyanidation (%)	93.0	92.5	93.4	93.9	96.6	97.1	97.5
Plant availability (%)	99	98	98	98	99	99	99

tpm: tonnes per months; g/t: grams per tonne; Concentrate S^{2-} grade (%) gives an indication the amount of reduced sulfur compounds present within the ore treated.

Gold is not the only metal mined by means of stirred tank biooxidation processes. Recently demonstration biooxidation plants have been constructed for the extraction of copper (BioCOP®) and nickel (BioNic pilot scale) while a commercial plant has been built for the recovery of cobalt (Dew et al., 1997, Briggs and Millard, 1997). Most of the biooxidation plants were built by BHP-Billiton (previously Gencor) and operate at 40°C (Dew 1995, Dew et al., 1997). A plant at the Youanmi mine in Western Australia designed by BacTech operates at 50°C (McCready, 1988) and contains a different consortium of organisms when compared to the plants operating at 40°C.

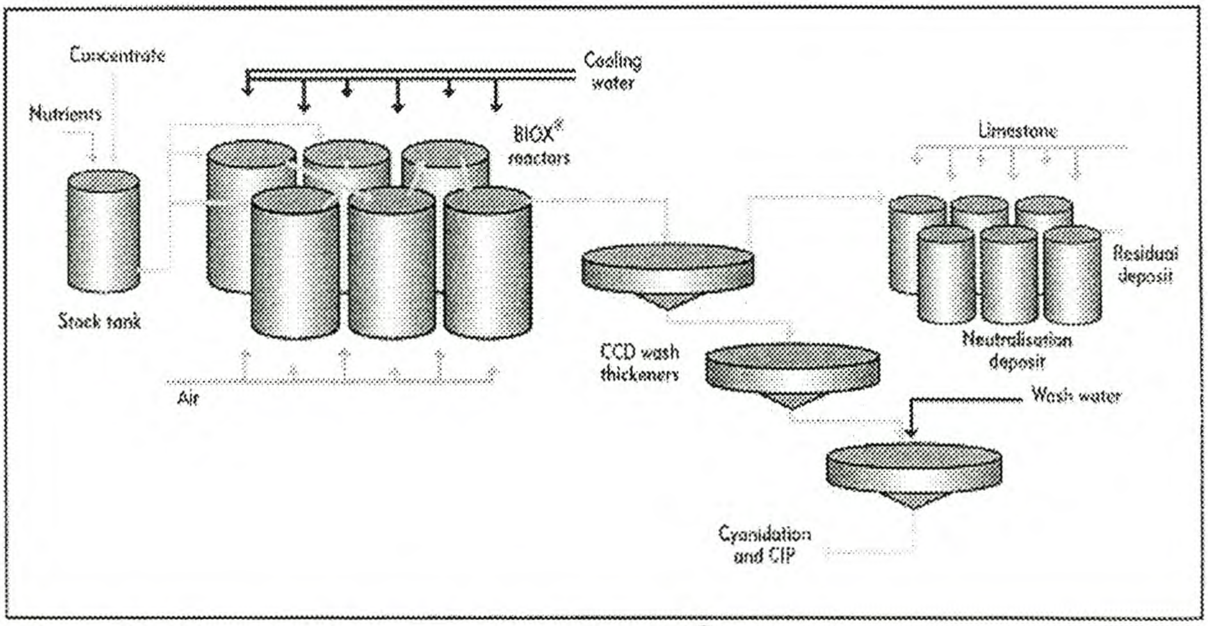

Figure 1.2 Diagrammatic representation of the BIOX® process

1.3.2 BioCOP® process

The BioCOP® process generally involves the use of microbes for the oxidation of copper sulfide concentrates in agitated reactors at temperatures of 40°C-45°C (mesophillic oxidation) or 65°C-85°C (thermophillic oxidation). After bioleaching, the slurry that contains soluble copper sulfate undergoes a process wherein the liquid and solids are separated and the slurry washed. Copper is subsequently extracted by means of cementation or SX/EW processes and the barren solution recycled in order to meet the acid requirements of the leaching concentrate. Figure 1.3 is a diagrammatic representation of this process.

The process operating at temperatures reaching 75°C, to extract copper from chalcopyrite ore, is presently undergoing trials. The elevated temperature is necessary because the rate of chalcopyrite decomposition at normal biooxidation temperatures (40°C and 50°C) occurs too slowly. The reason for the slow decomposition is that chalcopyrite is more stable than ores like chalcocite, from which copper is normally extracted (Rawlings, 2002). It has also been shown that organisms (such as *Sulfolobus*) flourish at high temperatures and can readily contribute to the attack and decompose this highly stable mineral (Brierely C.L., 1982).

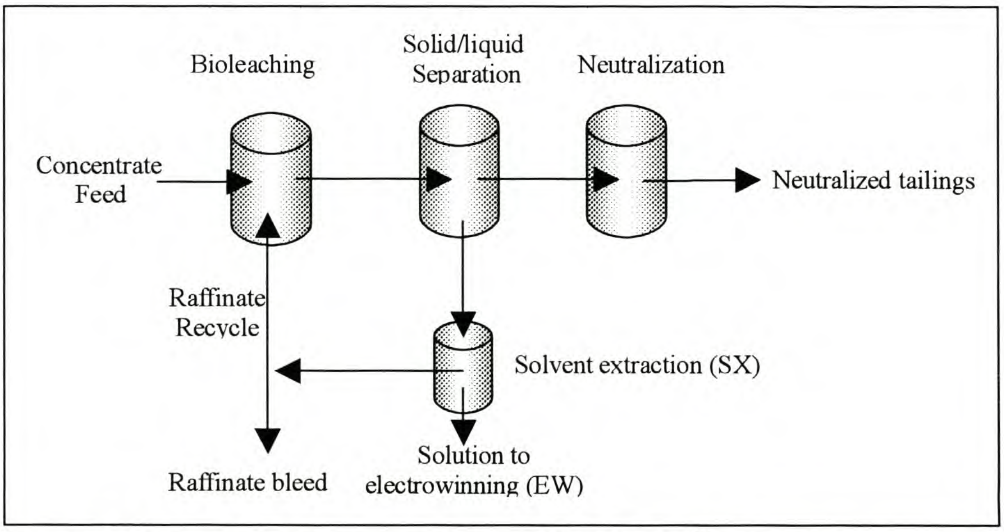

Figure 1.3 Diagrammatic representation of the BioCop® process.

1.4 Biooxidation mechanism

Lacey and Lawson (1970) showed that the ferrous iron oxidation rate was increased more than a million fold when *At. ferrooxidans* was present, compared with the chemical oxidation of ferrous iron by dissolved oxygen with no bacteria present. The ferric iron produced during the oxidation processes has the ability to chemically oxidize sulphide minerals. There has been a long-standing debate about the type of mechanism that the bacteria employ during metal oxidation. The proposed type of mechanism is either the direct or indirect mechanism (Lundgren and Silver, 1980). One of the reasons for the debate concerning the type of mechanism employed is the lack of clarity about what exactly is meant by "direct" and "indirect".

1.4.1 Indirect bacterial leaching

This mechanism of leaching entails the chemical attack by ferric iron or protons on a mineral sulfide. Ferric iron is a very powerful oxidizing agent that reacts strongly with other metals. This interaction often results in the solubilization of the metal within an acid environment. The role of the biomining bacteria is to oxidize ferrous iron (Fe^{2+}) to the more reactive ferric iron (Fe^{3+}) (Crundwell, 1997) and to maintain a high redox potential within the environment (Hansford, 1997).

1.4.2 Direct bacterial leaching

What is understood by direct leaching is not always clear. Two models have been proposed: one in which the bacterium is in direct contact with the mineral, and a second in which the bacterium is only in close proximity to the mineral. It would, therefore, be better to use the term "contact leaching" in place of direct leaching (Rawlings, 2002). What contact or direct leaching loosely means is that the attachment of the bacteria to the ore has an enhancing effect on the oxidation process. In the direct leaching mechanism this is accomplished by an enzymatic attack by the bacteria on those components within the mineral that are susceptible to oxidation (Brierely C.L., 1982, Sand et al., 1995).

1.4.3 Most probable leaching mechanism

The current understanding is that leaching mechanisms are indirect, although proximity of cells to the mineral surface can enhance leaching rates. Sand and Tributsh emphasized the importance of the exopolysaccharide or extracellular polymeric substance (EPS) layer during metal dissolution (Gercke et al., 1998). This EPS layer acts as a reaction space in which the ferric iron concentration increases significantly compared to the concentrations within the surrounding areas. As mentioned earlier it is the action of ferric iron on the mineral, which results in the dissolution of the metal. During the oxidation of the mineral, ferric iron within the EPS layer is reduced to ferrous iron. A microbial reoxidation of the ferrous iron results in a localized pH increase within the EPS layer. Some researchers have postulated that it is this increased pH, which aids the mineral dissolution (Fowler, 1999 and 2001, Holmes, 1999). Tributsch (2001) noted that during the oxidation of pyrite a drastic increase of colloidal sulfur within the EPS is present. At first this appeared to be a wasteful process until it was noted that these sulfur colloids and intermediates are used as an energy source for other sulfur-oxidizing bacteria. This process can be seen as a cooperative leaching interaction.

Another important observation made by Sand and co-workers is that the oxidation of different metal sulfides proceeds through different intermediates (Schippers et al., 1996, Schippers and Sand, 1999). Two categories of metal sulfides have been proposed namely those that are acid insoluble and those that are acid soluble. The solubility of the metal sulfides is determined by whether or not the valence bands contribute to the chemical bond between the metal and the sulfur moiety within the crystal (Schippers and Sand, 1999). The sulfides in which both the metal and sulfur orbitals contribute towards the valence bands are found to be acid soluble and have thiosulfate as an intermediate. The polysulfide mechanism of oxidation has elemental sulfur as its main intermediate and utilizes acid insoluble sulfides. The thiosulfate mechanism relies solely on the oxidative attack of ferric iron (Fe^{3+}) ions on the acid insoluble metal sulfates such as FeS_2 (pyrite), MoS_2 (molybdenite) and WS_2 (tungstenite). The polysulfide mechanism however relies on either ferric iron (Fe^{3+}) ion and/or proton attack of metal sulfates such as ZnS (sphalerite), $CuFeS_2$ (chalcopyrite) and PbS (galena). These mechanisms may be simplified by the following equations (Schippers, 1999):

Thiosulfate mechanism

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+$$

Polysulfide mechanism (metal sulfides: MS)

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5H_2S_n + Fe^{2+}\ (n \geq 2)$$

$$0.5H_2S_n + Fe^{3+} \rightarrow 0.125S_8 + Fe^{2+} + H^+$$

$$0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$$

It can therefore be said that the role of the biomining bacteria in the oxidation of metal sulfides is to generate acid biologically and to keep the iron in the oxidative (ferric) state. The acid will provide protons for a hydrolysis attack and the ferric iron will take part in an oxidative attack on the mineral (Schippers and Sand, 1999) (Fig.1.4).

Another proposed mechanism of mineral dissolution was suggested recently by Tributsch (2001). He proposes that the breakdown of the crystal structure of the mineral can be because of one of five reasons:

(a)reaction of protons with sulfides in order to release SH^- ions;

(b)the extraction of electrons from the valence bands from sulfides which will result in the release of metal ions and sulfur compounds;

(c)already present broken chemical bonds which will result in higher interfacial dissolution;

(d)reaction with metal complex-forming or polysulfide agents; or

(e)electrochemical dissolution that will occur because of continual electron extractions and depolarization of the mineral, which normally occurs at high concentrations of ferric irons.

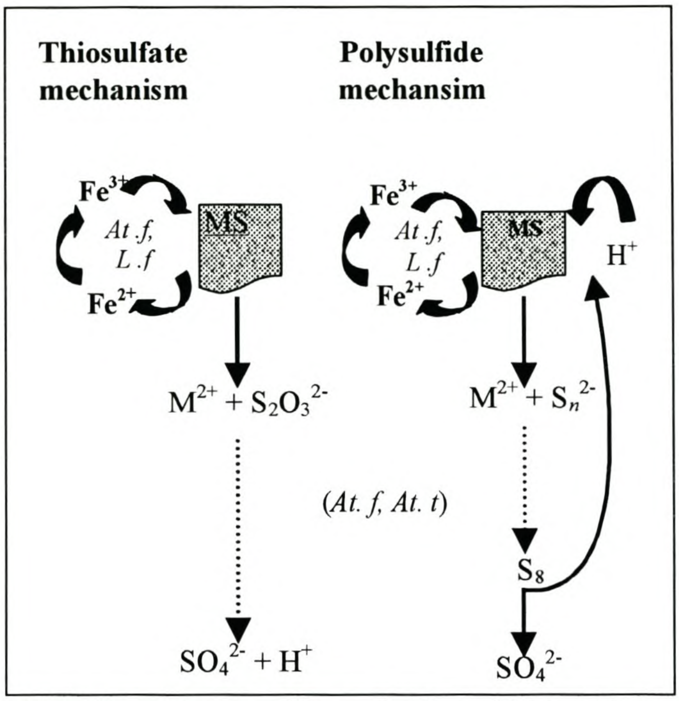

Figure 1.4 The thiosulfate and polysulfide mechanism of bioleaching. The properties of the metal sulfides (MS) play a big role in which mechanism is followed. The dashed line indicates the occurrence of intermediate sulfur compounds. *At. f*: *At. ferrooxidans*; *At. t*: *At. thiooxidans*,; *L. f*: *L. ferrooxidans* (Adapted from Schippers and Sand, 1999).

It is quite clear that the first three reasons are comparable to the indirect leaching mechanism. Tributsch (2001) further postulated that processes that require the presence of a sulfur carrier (d) or highly elevated ferric iron concentration (e) rely on the close proximity or direct contact of the bacteria to the mineral.

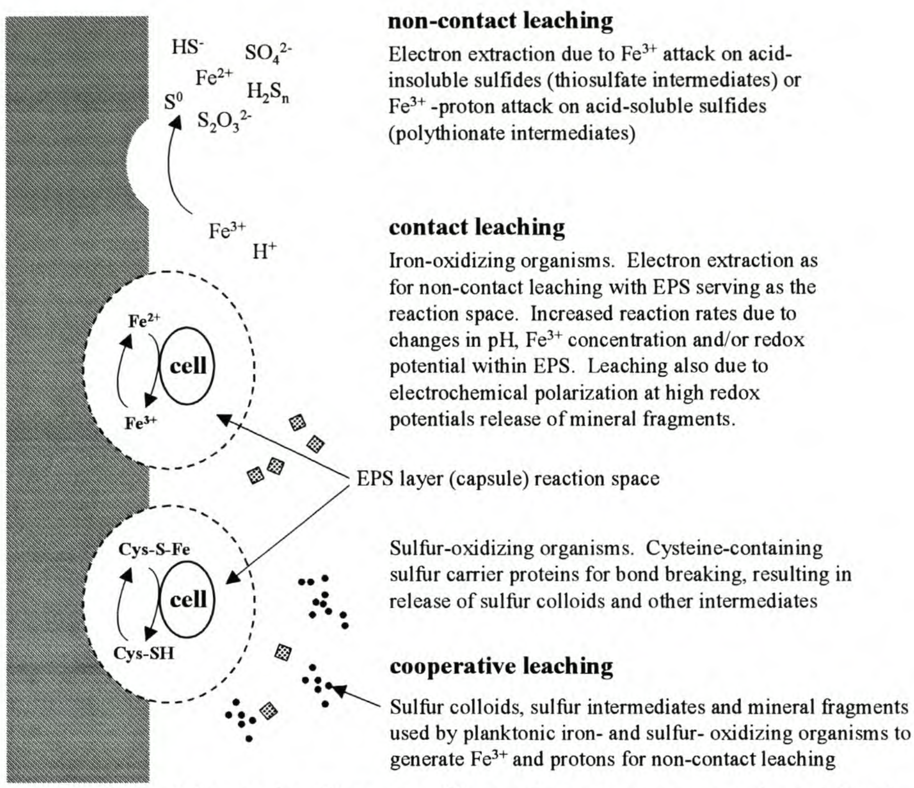

Figure 1.5 Schematic diagram illustrating the proposed mechanisms of pyrite biooxidation (Rawlings 2002)

At present no consensus has been reached concerning "direct" vs. "indirect" leaching. The best approach may be to consider mineral solubilization as due to indirect leaching ("non-contact" leaching) but with certain types of solubilization being drastically enhanced by the close proximity ("contact" leaching) of the bacteria and the mineral surface. Figure 1.5 illustrates how the two biooxidation mechanisms namely direct ("contact") and indirect ("non-contact) leaching work together.

1.5 Microorganisms in biomining

Organisms isolated from areas where mineral oxidation occurs are divided into three groups according to their preferred growth temperatures. The mesophiles have an optimal growth temperature range of 25°C-40°C, the moderate thermophiles an optimal growth temperature range of 40°C-60°C, and the extreme thermophiles an optimal growth temperature range of 60°C-80°C of which certain species maintain activity at temperatures as high as 85°C. The habitat of these latter bacteria is very inhospitable to most microorganisms on account of the extremely low pH, elevated presence of inorganic minerals, and metals and the above average temperatures.

Biomining organisms are chemolithoautotrophic, meaning that they derive their energy from inorganic substances and minerals. It is important to note that the inorganic ions never physically enter the bacterial cell. During the oxidation reaction electrons are released at the surface of the cytoplasmic membrane. These electrons pass through the cell membrane via an electron transport system and are passed on to oxygen when the bacteria are respiring aerobically. Several of these organisms have the capacity to respire anaerobically with ferric iron being the final electron acceptor. Neutralization of the cytoplasm is required due to the entry of protons, which is in turn coupled to the formation of adenosine triphosphate (ATP), the "energy currency" of the cell.

Iron oxidising bacteria make use of the electrons transferred during the oxidation of ferric iron (Fe^{2+}) to ferrous iron (Fe^{3+}) whereas the sulfur oxidising bacteria derive their energy from the oxidation of elemental sulfur to sulfuric acid.

Iron oxidising bacteria:

$$4Fe^{2+} + 4H^{+} + O_2 \xrightarrow{\text{BACTERIA}} 4Fe^{3+} + 2H_2O$$

Sulfur oxidising bacteria:

$$2S + 3O_2 + 2H_2O \xrightarrow{\text{BACTERIA}} 2H_2SO_4$$

1.6 Mesophillic bacteria

1.6.1 *Leptospirillum* species

Leptospirilla are Gram-negative obligate chemolithoautotrophic bacteria with vibrio or spiral shaped cells depending on the growth stage of the culture (Fig.1.6). Their optimum pH lies between 1.3 – 2.0 and their optimal growth can either be mesophillic (between 30°C-40°C) or moderately thermophillic (between 45°-55°C) depending on the species (Coram and Rawlings, 2002). Three Leptospirilla species have so far been identified, two of which (*L. ferriphilum* and *L. thermoferooxidans*) can be classified as moderately thermophillic (Coram, 2002, Schrenk, 1998, Hippe, 2000) and the third, *L. ferrooxidans*, being mesophillic (Hippe, 2000, Markosyan, 1972.). They are motile with a polar flagellum. (Coram and Rawlings, 2002). When grown on solid media containing ferrous iron, brown colonies with large red halos are produced due to ferric iron production (Battaglia et al., 1994).

It has recently been proven that Leptospirilla are more important to natural as well as deliberate mineral bioleaching and biooxidation processes than what was previously perceived (Coram and Rawlings, 2002). Leptospirilla have been shown to be the dominant iron-oxidizing bacteria found in continuous flow biooxidation tanks, rather than *Acidithiobacillus ferrooxidans* as was previously thought. Two of the major reasons why Leptospirilla are more prevalent than *At. ferrooxidans* are their ability to withstand higher ferric-ferrous iron concentrations and lower pH values (Coram and Rawlings, 2002, Schrenk et al., 1998, Battaglia et al., 1972).

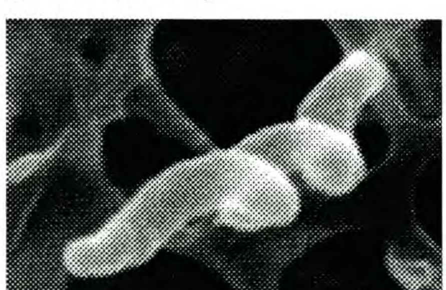

Figure 1.6 Scanning electron micrograph depicting *Leptospirillum ferrooxidans* (Adapted from Rawlings, 1997)

1.6.2 *Acidithiobacillus* species

1.6.2.1 *Acidithiobacillus ferrooxidans*

At. ferrooxidans was the first biomining microorganism to be isolated (previously called *Ferrobacillus ferrooxidans* and then *Thiobacillus ferrooxidans*) and is the most studied of all the biomining bacteria. Until recently it was reputed to be the most abundant organism found in the biooxidation processes. *At. ferrooxidans* is an obligate heterotroph that derives its energy from either the oxidation of ferrous iron or from reduced sulfur compounds. It has been shown that organic compounds are inhibitory to its cellular growth (Rawlings and Kusano, 1994). *At. ferrooxidans* is a Gram-negative, rod shaped, non-motile bacterium with an optimal growth pH between 1.5 and 2.5. The optimal growth temperature lies between 30°C and 35°C.

The major contribution of *At. ferrooxidans* to the metal extraction process is its ability to attack sulfide minerals and convert insoluble sulfides into soluble metal sulfates (Rawlings and Kusano, 1994). *At. ferrooxidans* is thought to have two predominant roles in the dissolution of pyrite minerals namely (1) catalysing the oxidation of ferrous ions to regenerate ferric iron (pyrite is dissolved by ferric iron) and (2) enhancing the rate of pyrite oxidation (Silverman and Ehrlich, 1964). The latter is accomplished by increasing the pH level at the mineral surface (Fowler et al., 1999 and 2000). The main contribution of *At. ferrooxidans* to the corrosion system is to maintain a stable ferric iron concentration (Gehrke et al., 1998). Extracellular polymeric substances (EPS) are excreted by *At. ferrooxidans* and mediate contact with the sulfidic energy source. This in effect creates a larger area for the cells in which to extend their reactions (Gehrke et al., 1998). One of the features that distinguishes *At. ferrooxidans* is its inherent resistance to high concentrations of metallic and other ions and its high adaptability to adverse environments (Rawlings and Kusano, 1994). *At. ferrooxidans* has a higher iron oxidation rate than *L. ferrooxidans*, but as mentioned earlier, *Leptospirillum* has a higher ferric-ferrous iron tolerance than *At. ferrooxidans* and is therefore found to be more prevalent within biomining environments in which the steady-state ferric iron concentration is high

(Coram and Rawlings, 2002, Schrenk et al., 1998, Battaglia et al., 1972). Metal and ion resistance is possibly plasmid encoded.

1.6.2.2 *Acidithiobacillus thiooxidans*

At. thiooxidans is a rod shaped, Gram-negative obligate autotroph. Unlike *At. ferrooxidans*, it is unable to utilize ferrous iron as an electron donor, relying solely on the oxidation of reduced sulfur compounds for its energy needs. It has an optimal growth temperature between 30°C and 35°C and its pH range extends much lower than any of the other acidophillic biomining organisms. Growth has been noted at pH values below 0.8 (Norris, 1983). The heavy metal resistance of *At. thiooxidans* is much lower than that of both *At. ferrooxidans* and *L. ferrooxidans*. Because *At. thiooxidans* is not able to oxidize ferrous iron, no ferric iron can be produced and, therefore, *At. thiooxidans* is not able to solubilize minerals that are not acid soluble. When *At. thiooxidans* is grown in combination with *At. ferrooxidans* or *L. ferrooxidans* however, it helps improve the overall mineral oxidation by removing sulfur deposits from the mineral surface (Rawlings, 1997).

1.7 Moderately thermophillic bacteria

1.7.1 Acidithiobacillus caldus

At. caldus is a moderately thermophillic, acidophillic bacterium. It was placed within the genus *Acidithiobacillus* based upon its ability to utilize reduced sulfur compounds as electron donors for growth, and its ability to flourish at extremely low pH values. This placement has been confirmed using 16S rDNA sequence data (Hallberg & Lindström, 1994). The optimum pH conditions for *At. caldus* are between pH 2.0 and pH 2.5 and given a suitable sulfur source, cells double in approximately 2.3 hours. They are Gram-negative, short rod-shaped bacteria that are often found in pairs. They are motile and have a polar flagellum (Hallberg and Lindström, 1994). It has also been shown that *At. caldus* has an optimum temperature of 45°C when grown on sulfur and sulfide minerals.

At. caldus was found to be the most common sulfur-oxidizing bacteria isolated from continuous biooxidation reactors operating between 40°C and 50°C. It has been shown that *At. caldus* is a close relative of *At. thiooxidans* (Rawlings et al., 1999). *At. caldus* are found in mixed cultures, either with *L. ferrooxidans* or *Sulfobacillus thermosulfidooxidans*, where the combination ensures the optimal oxidation of the minerals (Norris, 1997).

It has been proposed that *At. caldus* has three roles within the leaching environment (Dopson, Lindström, 1999), the first and most important being the removal of solid sulfur from the surface of the mineral. A sulfur buildup on the mineral surface has an inhibitory effect on the bioleaching ability of the biomining microorganisms as it has been shown that direct microbial attachment may be necessary for optimal leaching (Arredondo et al., 1994, Ahonen and Tuovinen, 1995). The second putative role of *At. caldus* during bioleaching is to release organic chemicals, or growth factors. These (organic) growth factors may stimulate heterotrophic and mixotrophic growth by means of cross feeding (Borichewski, 1967). The third postulated role involves the solubilization of solid sulfur by the production of surface-active agents. These surface-active agents act as wetting agents that allow S^0 to be dispersed in the media, thereby allowing further oxidation of the mineral.

1.7.2 Sulfobacillus

Sulfobacilli have been isolated from various environments such as mineral sulfide mines, coal and mineral spoil heaps, geothermal environments and commercial metal leaching dumps where they have been shown to oxidize iron (Fe^{2+}), sulfur (S^0) and sulfide compounds.

1.7.2.1 Cellular morphology

Sulfobacillus is a Gram-positive bacterium and it is very similar to *Bacillus* in its capacity for sporulation and cell ultrastructure (Golovacheva and Karavaiko, 1978). Spores produced by Sulfobacilli are either oval or round and can be found terminally, subterminally or paracentrically (Fig. 1.7). Endospores are normally found when energy sources are low especially when the cultures are grown autotrophically in the presence of yeast extract. In contrast to most Bacilli, Sulfobacilli have no flagellar apparatus. Limited motility has been noted for Sulfobacilli when grown autotrophically on ferrous iron (Golovacheva and Karavaiko, 1978, Norris, et al, 1996). Another difference between Sulfobacilli and the Bacilli is that Sulfobacilli form structures similar to pili (fimbria) or slime sheaths through which they adhere to the surfaces of the substrates during oxidation (Kovalenko and Malakhova, 1982). *S. acidophilus* is known to produce distorted cells when grown in the presence of ferrous sulfate, most likely because of the increased acidity of the media. Another distinctive difference between *Sulfobacillus* and *Bacillus* is the ability of the former to have a wide range of cell morphologies, which exceeds the limits of range of possibilities for the *Bacillus* genus. It was shown by Golovacheva (1978) that Sulfobacilli are found primarily as straight rodlike cells, but that coccoid, pyriform and clavate forms are also present. Another interesting finding by the same workers was the capacity of Sulfobacilli cells to branch. It was also shown that separation of cells during cell division could occur before rounding, which results in daughter cells with the illusion of lens shaped apical caps. Dividing cells often do not separate properly from each other with the consequent formation of bent chains, palisades, rings and Y-formations (Golovacheva, 1978). Transformation of rodlike cells into coccoid, pyriform and clavate cells; cell division by sudden snapping; formation of cell aggregates in the form of palisades, Y-formations and more complex combinations and cell branching are all typical features of coryneform bacteria (Bergey's Manual, 1974).

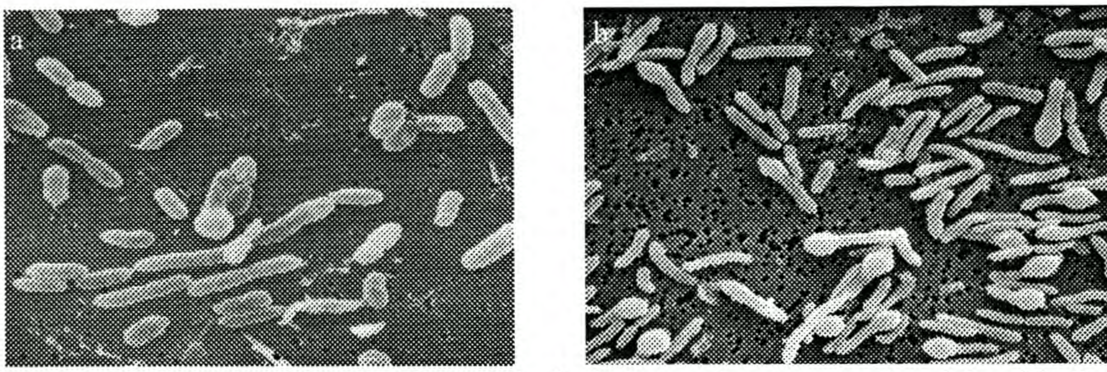

Figure 1.7 Scanning electronmicrograph depicting endospores produced by (a) *S. monseratensis* and (b) *S. ambivalens* (unpublished data Barry Johnson)

It has been suggested that the genus *Sulfobacillus* may have originated from a heterotrophic bacterial genus that is similar to it in morphological properties. This hypothetical genus could have been represented as a transitional form between coryneform bacteria and *Bacillus* or between actinomycetes and *Bacillus* (as the actinomycetes are closely related to coryneform bacteria). The latter scenario is the most appealing since it would fill the gap in the phylogenetic relationships between Bacilli and actinomycetes (Golovacheva, 1978).

1.7.2.2 Fatty acid composition

Another distinguishing characteristic of Sulfobacilli is the unique fatty acid composition of their lipids. All bacteria are divided into three groups according to the lipid composition of their cell membranes. Group II includes bacteria whose membranes consist of branched-chain and alicyclic acids (Kaneda, 1991). Only about 10% of all known bacteria are included within this group. According to Tsaplina et al. (1994), Sulfobacilli belong to this group of organisms. A prevalence of branched-chain fatty acids in Sulfobacilli's lipids, groups it with bacteria that use short-chain branched precursors of fatty acid synthesis (α-keto acids or short-chain fatty acids) for lipid production. This distinguishes Sulfobacilli from most other living organisms that produce straight-line saturated and unsaturated fatty acids using short-chain acetyl-CoA esters as primers and malonyl-CoA for chain elongation.

Another unique group of lipids synthesized by Sulfobacilli are the ω-cyclohexane acids. These acids have only been detected previously for the acidothermophiles *Alicyclobacillus acidocaldarius* and *A. acidoterrestris* (Oshima and Ariga, 1975; Kaneda, 1991), and the mesophile *Curtobacterium pusillum* (Suzuki et al., 1981). The Sulfobacilli studied also contained ω-cyclohexyl-α-oxyundecanoic acid. This acid has never before been found in any bacteria, including those synthesizing ω-cyclohexane acids (Tsaplina et al., 1994). Lipids containing ω-cyclohexane acids are very tightly packed, which results in a decrease in diffusion at high temperatures. This is possibly one of the mechanisms employed by the organism to survive elevated temperatures. Linear α-oxyacids, also found in Sulfobacilli's fatty acid profile, suggest an ability to synthesize sphingolipids (Goldfine, 1982; Yano, 1985). Sphingolipids are very rarely found in prokaryotes but may also play a role in rendering the cells resistant to certain environmental effects (Tsaplina et al., 1994). This unique lipid composition of *Sulfobacillus* spp. can be used as an identification tool.

1.7.2.3 S. thermosulfidooxidans* and *S. acidophilum

At present there are only two recognized species within the genus, namely *S. thermosulfidooxidans* and *S. acidophilus* (Golovacheva, 1979; Norris et al., 1996). The two species have been differentiated phylogenetically as well as with the aid of 16S rDNA sequence analysis. The genomic DNA of *S. thermosulfidooxidans* and *S. acidophilus* species have a guanine-cytosine (GC) content of 52-54 mol % and 55-57 mol. %, respectively. *S thermosulfidooxidans* and *S. acidophilum* have an optimal growth temperature of between 45°C and 50°C with a temperature maximum of approximately 60°C. Their optimal growth pH lies between 1.5 and 2.5.

S. thermosulfidooxidans is further divided into two subspecies namely *S.th.* subspecies *thermotolerance* and *S.th.* subspecies *asporogens*. The basic characteristic that distinguishes *S.th.* subspecies *asporogens* from *S.th.* subspecies *thermotolerance* is the absence of spores. There is also a slight difference in the molecular weight of their genomes (3.7 x 10^9 D in *S.th* subspecies *thermotolerance* and 3.0 x 10^9 D in *S.th.*

subspecies *asporogens*). This decrease in the genome size has been described in several cases in which Bacilli lose their ability to produce spores (Varanyan et al., 1986). *S.th.* subspecies *thermotolerance* differs from *S.th. subspecies asporogens* in respect of its optimal and maximum growth temperatures. The morphology of the *S.th. subspecies thermosulfidooxidans* is stable between temperatures of 30°C and 42°C, but giant, distorted cellular forms, with long filaments, appear at temperatures above 50°C. The organism is more thermotolerant than thermophillic, thus the name *S.th.* subspecies *thermotolerance* (Kovalenko and Malakhova, 1982). Another slight variation between *S thermosulfidooxidans* and *S.th.* subspecies *thermotolerance* is the position of the spores. The spores of the latter have a terminal position whereas the type culture's spores are either sub-terminal or central.

1.7.2.4 Autotrophic, heterotrophic and mixotrophic growth

Both species of Sulfobacilli are capable of growth on solid media. Colonies produced on media containing iron are round and smooth edged. As these colonies start to age they become encrusted with iron oxide hydrates, the color ranging from bright yellow to deep reddish brown (Fig.1.8) (Golovacheva and Karavaiko, 1978). In liquid media with sulfide minerals and elemental sulfur (S^0), *S. thermosulfidooxidans* and *S. acidophilum* oxidize the sulfur to sulfuric acid, which results in a rapid acidification of the media. *S. thermosulfidooxidans* is unable to maintain autotrophic growth on sulfide minerals when no yeast extract is present, whereas *S. acidophilus* has the capability of maintaining autotrophic growth on sulfide minerals over several generations even in the absence of yeast extract (Golovacheva, 1979 Norris et al., 1996, Varanyan et al., 1986).

When grown heterotrophically on yeast extract as the sole energy source, *S. acidophilus* has a mean doubling time of 6-8 hours and *S. thermosulfidooxidans* 8 - 12 hours. S. *thermosulfidooxidans* is the more active species when it comes to the oxidation of ferrous iron, whereas *S. acidophilus* is more active in the oxidation of sulfur. Both of the species are capable of mixotrophic growth on ferrous iron (Golovacheva, 1978, Norris et al., 1996). Slight ferrous iron end-product inhibition has been noted for both *S.*

thermosulfidooxidans and *S. acidophilum* with the latter showing more sensitivity. Cells become elongated when grown autotrophically at low acidity and during growth on some concentrate samples (Norris, 1997).

A further four putative species have been identified namely, *S. yellowstonensis*, *S. ambivalens*, *S. montseratensis* and *S. sibiricus* (unpublished data NCBI database). *S. ambivalens* and *S. montserratensis*, isolated from the volcanic Caribbean island of Montserrat, show optimal growth temperature within the mesophillic range. Their pH tolerance is greater than any previously described acidophillic iron-oxidizing bacteria, with growth possible at pH values as low as 0.7.

A B

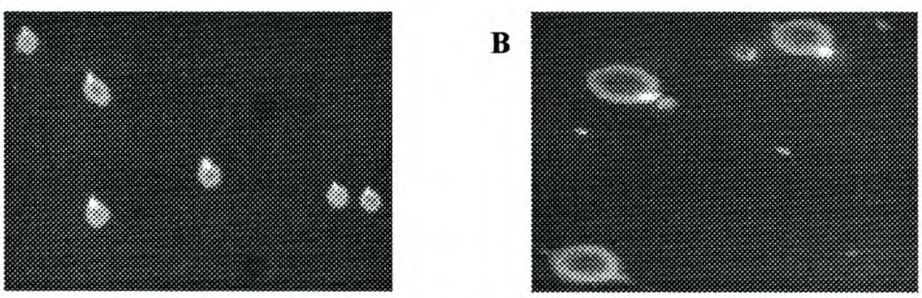

Figure 1.8 *S. ambivalens* (A) and *S. monseratensis* (B) colonies on SJH-overlay plates. Clear precipitations of iron oxides are visible as an orange to red color (unpublished data Barrie Johnson).

Early reports indicated that Sulfobacilli have the ability to respire anaerobically using ferric iron as the final electron acceptor with either an organic (i.e. glycerol) or inorganic (i.e. tetrathionate) electron donor .

1.7.3 Alicyclobacillus spp.

Alicyclobacilli are Gram-positive, spore-forming, rod-shaped moderately thermophillic obligate acidophilic heterotrophs. They have been found in wide ranging environments from orange juice, to geothermal sites within the Yellowstone National Park (Hallberg and Johnson, 2001). There are no reports of *Alicyclobacillus* spp. being isolated from mining environments although some species have been shown to flourish when maintained in mixed culture with certain *Sulfobacillus* spp. (Johnson et al, unpublished data). Novel *Alicyclobacillus* spp. have been identified that have the ability to reduce ferric iron, although it is not known if the reduction is capable of sustaining growth within an anoxic environment. A characteristic feature of Alicyclobacilli is the presence of ω-alicyclic/ω-cyclohexane fatty acid as their major cellular lipid component (Hallberg and Johnson, 2001).

Four species of Alicyclobacilli are presently recognised namely *Alb. acidocaldarius*, *Alb. acidoterrestris*, *Alb. cyclohepatanicus* and *Alb. hesperidum*. In 1996 a novel *Sulfobacillus* spp. (*S. disulfidooxidans*) was proposed by Dufresne et al. (1996). Phylogenetically this organism was found to be more closely related to *Alicyclobacillus* spp. than to *Sulfobacillus* spp. and it has subsequently been shown that the classification of this organism as a member of the genus *Sulfobacillus* was invalid. Hallberg and Johnson (2001) propose that *S. disulfidooxidans* should be reclassified as *Alb. disulfidooxidans*, not only because of its 16S rDNA sequence similarity but also because of the presence of the diagnostic ω-alicyclic fatty acids.

1.8 Thermophillic organisms

Thermophiles are organisms that flourish at temperatures where life for most living organisms would be impossible. The majority of these organisms are archaea with only a few (strongly resembling *Alicyclobacillus* spp.) being bacteria. The thermophilic archea *Sulfolobus* and *Acidianus* are capable of heterotrophic growth at temperatures up to 70°C. *Sulfolobus metallicus* (isolated from coal spoil heaps and hot springs) has been utilized for sulfide mineral oxidation at high temperatures in several laboratories (Norris 1997). Another *Sulfolobus*-like organisms namely *Metallosphaera sedula* has been identified, but will not be discussed in this work.

The chemistry of bioleaching occurs at a much faster rate at high temperatures and for this reason the mining industry is very interested in microorganisms that can tolerate these elevated temperatures.

1.9 Taxonomy introduction

For centuries scientists have aimed at creating a common language that they could use in order to describe all living organisms. The first classification system as described by Linnaeus during the 18th century, was based on morphological comparisons of the organisms being studied. This was the first natural classification system and was known as the morphospecies approach. This can be regarded more as an approach than an actual concept. The morphospecies concept worked very well for the classification of eukaryotes because they possess a large set of morphological features to use in the comparisons. As microbes have very limited morphological variation, their early classification tended to be rather less reliable. Another major factor that contributed to the inadequacy of the early prokaryotic classification was the lack of any fossil records to use in the comparisons.

Linneaus, the father of the binomial system of nomenclature and classification, suggested that a species was indivisible, thus being the basic taxonomic unit. The species is also arguably the most important and central element of bacterial taxonomy. The ranks above the species level (genus and higher classes) are often regarded as abstract and are thus easily comparable across a wide variety of classification systems (Van Rogenmortel, 1997). Species, however, are viewed as practical entities and the requirements for their circumscription is based upon the specific classification concept that is adopted (Van Rogenmortel, 1997).

The next approach, adopted during the early 1900s was Poulton's biological species concept. This was a phyletic or phylogenetic classification system that was based on the evolutionary relationship rather than the general similarity of existing characteristics. This concept emphasized the importance of interbreeding or potential interbreeding between organisms. Prokaryotes do not interbreed and the biological concept was thus not functionally acceptable for the prokaryotes. The best natural classification system for prokaryotes may be a phenetic system. This system groups organisms based on the mutual similarity of their phenotypic characteristics.

To date only about 5000 prokaryotic species have been described and classified. Microbial research might only have dealt with a very small portion of the Earth's prokaryotic diversity. Prokaryotes constitute a significant proportion of life's genetic diversity, catalyze unique and indispensable transformations in the biogeochemical cycles of the biosphere and produce important components of the Earth's atmosphere (Rosselló-Mora and Amann, 2001). The relatively low number (Fig. 1.9) of microbial organisms studied so far is due to the difficulties experienced with the isolation of many organisms in pure culture. Thus a very important step in microbiology was the development of techniques that allow the isolation of organisms in pure culture. Having the ability to work with organisms in pure culture leads to the direct retrieval of information about the organism (Logan, 1994) and is of major importance in bacterial classification.

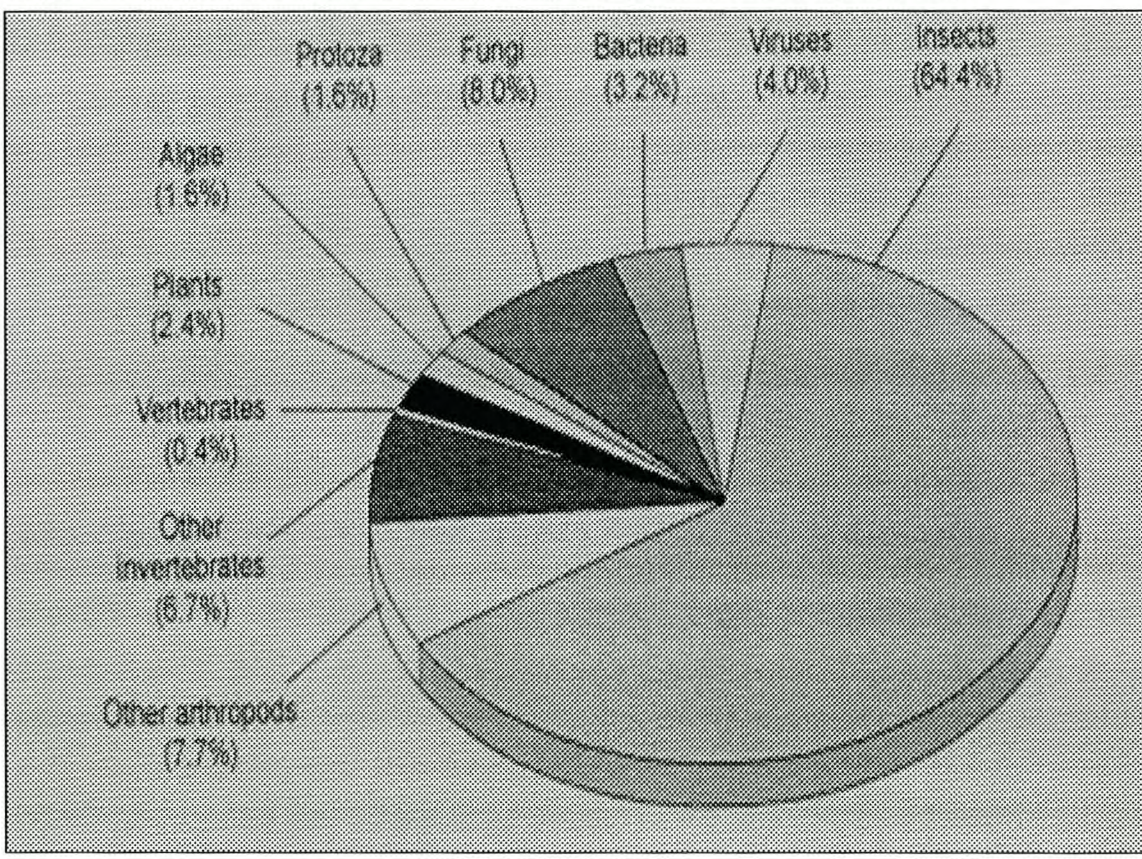

Figure 1.9 Estimates of proportions of species of major groups of organisms contributing to the total living biological diversity.

From as early as the 18th century to the middle of the 1950's there was no common agreement on prokaryotic classification. A large contributing factor to the confusing nomenclature was the lack of scientific exchange and the tendency to lean towards special purpose classification (Goodfellow and O'Donnell, 1993). During the 1950's a numerical classification system was born in conjunction with the development of the computer. The aim of numerical taxonomy was to devise a consistent set of methods for classification. This concept used mathematical procedures to compare a set of characteristics and group organisms according to their overall similarities. Numerical classification was very effective in delineating taxospecies but had less success in the construction of higher taxonomic ranks. Nevertheless, the strengths of numerical taxonomy far outweigh its limitations (Goodfellow et al., 1997).

In 1963, Ravin identified three realistic species definitions namely genospecies, nomenspecies and taxospecies. Genospecies includes mutually infertile forms and corresponds very closely with the biological species concept. Nomenspecies are made up of individuals that bear a resemblance to the nomenclatural type strain, and taxospecies are groups of strains that have a high proportion of common properties. A fourth species definition has recently been added to the list, namely the genomic species, and should not be confused with the genospecies. Genomic species are organisms that share a high DNA relatedness value (Goodfellow et al., 1997).

The term "taxonomy" has long been used as a synonym for systematics or biosystematics. Taxonomy is divided into three parts namely (1) classification, the orderly arrangements of organisms into taxonomic groups on the basis of similarities, (2) nomenclature, the labeling of the groups or units defined in (1), and (3) the identification of unknown organisms and the determination of which group or unit as defined in (1) and labelled in (2), to which they belong. It has been proposed that two additional parts can be added, namely phylogeny and population genetics. Phylogenetic relationships as determined by the 16S rDNA or 23S rDNA sequence data (and possibly other gene sets) effectively reflects the natural relationship between bacteria, and is a very important aspect of bacterial classification (Woese, 1987).

Population genetics gives a clear indication of the evolutionary relationship and genetic transfer between bacteria. Genetic material can pass between bacteria in two ways: (1) vertically from a mother to daughter cell during asexual binary fission or (2) horizontally by means of genetic transfer systems such as transduction, transformation or conjugation. Populations arising from vertical transfer are clonal and new alleles can only be passed to direct descendants. This is very limiting as no re-assortment of alleles for novel genetic combinations can occur. Horizontal transfer on the other hand can be seen as a method for sexual transfer of DNA that can result in the re-assortment of mutant alleles that leads to greater genetic diversity (Cohen, 1994). The exchange of new alleles is usually local and in the order of a few 100 bp and hence does not affect or disrupt the clonal population structure. It has been found that most of the genetic exchange that occurs is of genes that confer some adaptive advantage to the organism, and rarely of housekeeping genes (Selander et al., 1994).

Although horizontal gene transfer is a most common method for genetic exchange in prokaryotes (Lanka and Pansegrau, 1999), these exchanges occur less frequently than chromosomal recombination in eukaryotes but are very promiscuous (Ravin, 1963). If there is more than 2% sequence divergence among eukaryotes, genetic exchange is not possible. In contrast to this, genetic exchange can occur between prokaryotes with as much and possibly more than 25% sequence divergence (Cohan, 1996). If genealogical analysis is inferred from a single gene (such as the 16S rDNA gene), the question arises of whether we are dealing with a true organismal tree or only with a gene tree (Goodfellow et al., 1997). Recently, after full genome sequences of some organisms have been made accessible, some incongruities have appeared in the 16S rDNA derived phylogenetic trees of organisms (Gupta, 1998). This led to the conclusion that horizontal transfer may play a more important role in evolution than was originally perceived. The prokaryotic species concept as it stands at the moment is insensitive to genetic rearrangements. This is because the changing of the physical map of an organism will not markedly influence DNA hybridization. This implies that even if one of the phenotypic characters that is used in classification of the organism changes, the DNA similarity values will not be changed to a measurable extent.

There has been a need for an objective method aimed at the sorting of individual strains of bacteria into homogeneous groups (conventionally species), and arrangements of species into genera and higher groups (Sneath, 1989). During the 1960's this requirement was answered with the development of molecular biological techniques that made the classification of bacteria by whole genome comparisons possible. With the development of newer molecular techniques, such as DNA sequencing, the refinement of the current species concept was possible. Although the resolving power of rDNA sequence similarities is not sufficient to guarantee correct delineation of bacteria at the species level (Fox et al., 1992, Martinez-Murcia et al., 1992), it has been accepted that rDNA sequence analysis usually provides a stable and satisfactory framework for prokaryotic classification (Rossello-Mora and Amann, 2001).

It was found that combining as many different classification techniques as possible allows a more rigorous classification system for the lower taxonomic prokaryotic ranks such as the species level divisions to be attained.

1.10 Polyphasic classification approach

It is a consensus among modern day taxonomists that a reliable and stable classification can only be achieved when the genomic and phenotypic characteristics of the organisms are studied as thoroughly as possible (Fig.1.10). This is generally known as the polyphasic approach (Vandamme et al., 1996). Genomic information encompasses all the data that is accessible through the comparison of the nucleic acids of the organisms. Phenotypic data includes the visible or measurable expression of the interaction between the genotype and the environment.

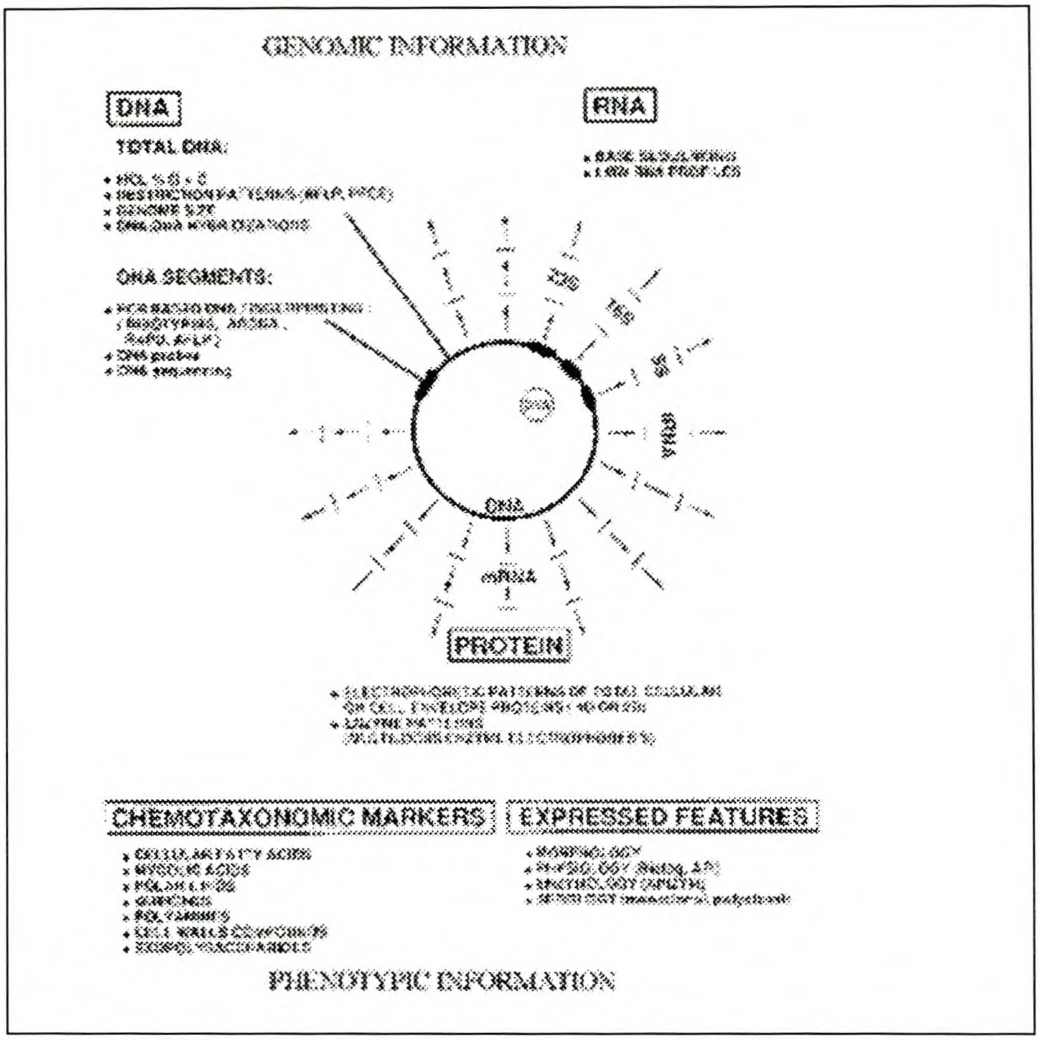

Figure 1.10 Schematic overview of various cellular components described and techniques used in species classification (Adapted from VanDamme et al.,1996)

1.10.1 Genomic information

A great advantage of using nucleic acids for classification systems is that environmental changes have no affect on the sequence of the DNA or RNA. During different growth phases only the amount of RNA present will fluctuate. Nucleic acids thus provide standard molecules by which a wide range of organisms can be compared. Genomic information can either be gained directly through sequencing or indirectly through methods such as DNA-DNA hybridization or mol% G+C values. Because it is not feasible yet to sequence the entire genomes of all the organisms being studied, alternative approaches to compare their genomes have been taken. These include techniques such as (1) determining the overall base composition (mol% G+C values) of the organisms, (2) comparing the genomic similarities by DNA-DNA hybridization, (3) generating sets of DNA fragments unique to the genome by using techniques such as pulse field gel electrophoresis (PFGE) and low-frequency restriction fragment analysis (LFRFA), (4) comparing sequences of selected genes, (5) DNA-rRNA hybridization and (6) sequencing of the rRNA genes.

1.10.1.1 DNA base composition (mol% G+C)

The DNA double helix is made up of four nucleotides namely guanine (G), thymine (T), cytosine (C) and adenine (A). Guanine and cytosine bind with one another by means of three hydrogen bonds, whereas only two hydrogen bonds connect thymine and adenine. The overall base ratio ([G+C]/[A+T]) is normally described as the abundance of the G+C pair. This value has proved useful in distinguishing between phenotypically similar but genomically different organisms (Goodfellow and O'Donnell, 1993). The base composition of organisms does not give any indication as to the linear sequence of the bases in the DNA. As a result of this, organisms with similar or identical base composition do not necessarily have very high sequence similarity. When two organisms have very dissimilar base compositions, they will also have very limited sequence similarity and are likely to be very distantly related. The overall base composition can thus be seen as a negative criterion. The more distantly related organisms are to one another, the greater their base compositions may differ.

Empirically it has been estimated that for organisms to be classified within the same genus they would have to have a base composition no different than 10-15 mol% G+C from one another (Goodfellow and O'Donnell, 1993). Base composition is also useful at species level where it has been suggested that organisms cannot belong to the same species if their mol% G+C differs by more than 5%. It is important to remember that similar G+C values only suggest close relationship if the organisms are also phenotypically similar and when other methods group them together.

There are two common methods of determining the mol% G+C value of an organism. The most commonly used method relies on determining the DNA melting temperature (T_m) of the organism. When DNA is heated slowly while spectrophotometric readings are taken at 260nm wavelengh, the strand separation can easily be followed. There is a sharp increase in the absorbance at 260nm as denaturation starts. The absorbance increase plateaus off when all the nucleic acid's double strands are separated from one another (Fig.1.11). The midpoint of this melting curve gives an indication of the melting temperature (T_m) of the organism. Nucleic acids that are more abundant in GC pairs have a greater number of hydrogen bonds. As a result of this, the strands will only separate at higher temperatures and their melting temperature will thus be higher. It is known that there is a linear correlation between the melting temperature and the GC content. The mol% G+C can be calculated by using the established empirical formula G+C = 2.44(T_m) – 169 (when mol% G+C determination occurs within a standard 0.1X SSC buffer).

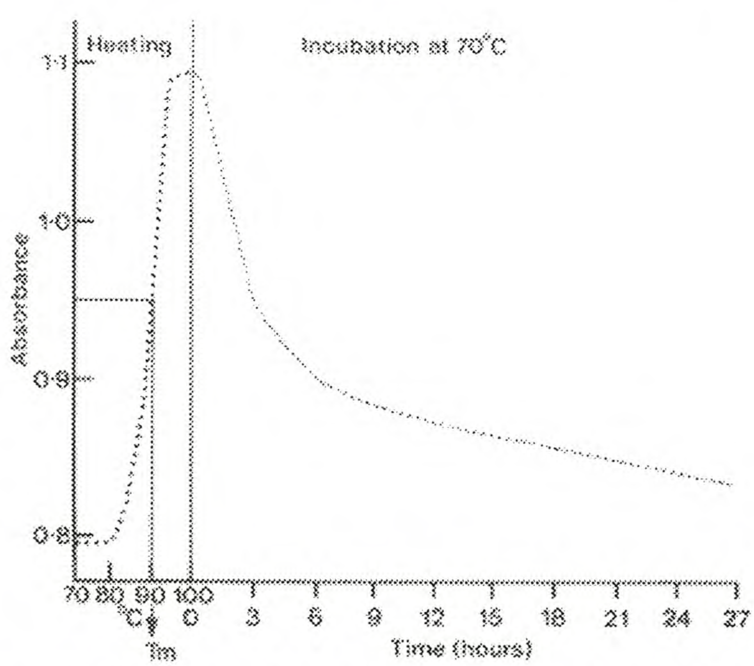

Figure 1.11 Denaturation and reassociation of *Nocardia farcinica* DNA. Absorbance was measured at 260nm. (Dradley and Mordarski, 1976).

The second, less used method makes use of the buoyancy factor of double stranded DNA. There is also a linear correlation between buoyant density and the GC content of DNA.

DNA of unknown density is run on a caesium chloride (CsCl) gradient. The sample DNA migrates within the CsCl gradient to a point in the gradient that is equal to its own density. The buoyant density of the unknown sample can now be calculated from the position of the band relative to the position of a standard DNA of a known buoyant density. The mol% G+C value is calculated as follows: Buoyancy density = 1.66 + 0.098(G+C). Both of these physicochemical procedures require the comparison of an unknown against a reference DNA, usually that of *Escherichia coli*.

1.10.1.2 DNA-DNA similarity (DNA-DNA hybridization)

The similarities between genomes can be compared more comprehensively by direct measurement of the similarities of the complete genome using nucleic acid hybridization. A characteristic property of DNA is its ability to reassociate after denaturation. Reassociation occurs because of hydrogen bond formation between the base pairs (A-T) and (G-C). The overall formation of double strand helices is dependent on the similar linear arrangements of the individual bases to one another. This is in direct contrast to the %mol G+C values that do not take the linear arrangement of the individual bases into consideration. The DNAs from different organisms will reassociate depending on the amount of similarity of their nucleotide sequences and by this it becomes possible to measure the amount of relatedness of the organisms. This is typically expressed as % similarity of the organisms. Scientists started using DNA-DNA hybridization techniques as taxonomic parameters when it was shown that there was a significant correlation between genomic DNA similarity and phenotypic similarity (Wayne et al., 1987).

There are several methodologies that measure DNA relatedness, but all of them rely on the same, very simple principle. The two most common ways of estimating DNA-DNA similarities are the free solution assay and the immobilized assay (Fig.1.12). A key feature of these techniques is that a large amount of single stranded DNA is incubated in the presence of a labeled probe (DNA from a different organism). The single stranded DNA is then allowed to reassociate to form double stranded molecules incorporating the probe DNA. The higher the genetic similarity between the organisms, the more nucleic acid bases they will have in common and the more hybridization will occur between them. The two major conditions by which the DNA relatedness is estimated are the relative binding ratio (RBR) and the difference in the thermal melting temperature (T_m) of heteroduplex and homoduplex DNA. Both of these criteria correlate and can be used independently in species circumscription (Stackebrandt and Goebel, 1994). The RBR gives an indication of the amount of heterologous DNA in comparison to the homoduplex DNA, which is considered to be 100% reassociation. This principle is normally used when utilizing the immobilized assay for determining the % DNA similarity.

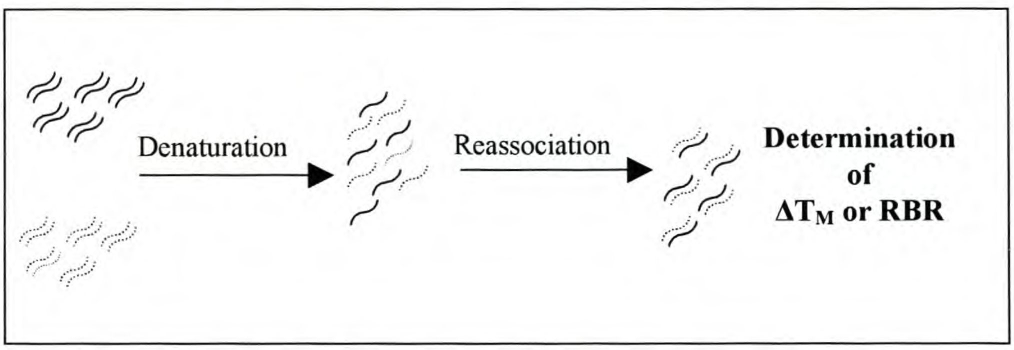

Figure 1.12 DNA-DNA reassociation assay in which DNA from two different organisms are denatured and mixed to give a solution of single stranded mixed DNA molecules. DNA reassociation occurs under controlled conditions with the degree of reassociation dependent upon the degree of genetic similarity. The degree of similarity is obtained by comparing the results obtained from a mixture of DNA and pure reference DNA with each other (Adapted from Rosellò-Mora and Amann, 2001)

When using the free solution assay the difference in the thermal melting temperature is normally used to estimate the % DNA similarity. There are three factors that influence the denaturation of double stranded DNA: the %mol G+C value, the ionic strength of the solution in which the DNA is dissolved and the thermal melting temperature (T_m). The only variable is the melting temperature as the ionic strength of the solution is known and because the %mol G+C value stays constant for every organism. The rate of denaturation is influenced by the amount of hydrogen bonds formed between the duplexes. Heteroduplexes will have a much lower amount of hydrogen bonding compared to homoduplexes, and are, therefore, much less stable, and will denature at a much lower temperature. The difference in the melting temperature (T_m) is the difference between the homoduplex T_m and the heteroduplex T_m (Fig.1.13).

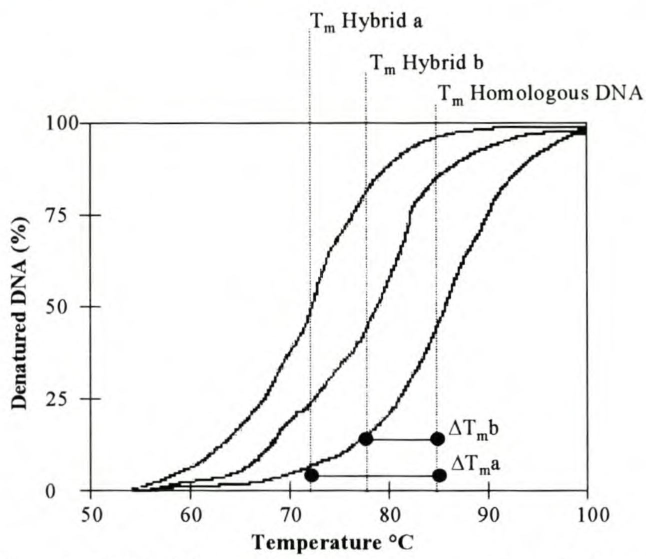

Figure 1.13 Thermal denaturation curve of homoduplex DNA and two heteroduplex DNA's (Reproduced from Roselló-Mora and Amann, 2001)

It is advantageous to use the free solution assay (T_m) method rather than the immobilized assay (RBR), as the latter has to take into consideration the differences that are related to the hybridization technique used (Grimont et al., 1980). Another advantage of the free solution assay is that data obtained from the T_m does not have to be processed any further.

It has been recommended that in order to define a species there has to be at least 70% similarity between the genomes of the organisms when measuring the RBR, and a 5°C or lower difference in their T_m values (Wayne et al., 1987). There are several disadvantages to using DNA-DNA hybridization studies in order to classify organisms. The most noteworthy is the fact that these studies are very time consuming because of pairwise comparisons that are needed. Because of the time constraint, experiments are normally carried out with a small data set and this can result in incorrect conclusions. Another negative factor is that it is postulated that hybrids will only form if there is at least an 80% complementarity between DNA strands (Goodfellow and O'Donnell, 1993). This limits the use of DNA-DNA hybridization to study closely related species. The advantages of DNA-DNA hybridization nevertheless greatly outweigh the disadvantages. The most prominent of the advantages is reproducibility, because the genome of organisms stays the same regardless of transient environmental conditions. Because this comparison makes use of the whole genome and not a small part as in phenetic studies, this is one of the most stable of the typing methods used.

1.10.1.3 rRNA analysis (nucleic acid sequencing)

The most comprehensive way in which to compare the genomes of organisms is by direct sequencing. Presently it is too costly and time consuming to sequence the whole genome of an organism, so the focus has fallen on sequencing a few conserved genes. The rRNA genes are ideal for phylogenetic comparisons and estimating evolutionary relatedness because they are essential to a critical organelle found in all living organisms (Woese, 1992). The role of rRNA is the same within all ribosomes and we can thus say that it has a universal, constant and highly constrained function. Their rate of mutation has also been shown to be very slow, most likely because of the critical role of rRNA and its requirement to interact with many proteins and RNAs of the ribosome complex. Another positive aspect is that hardly any lateral gene transfer has occurred between rDNA genes (Goodfellow et al., 1997).

Prokaryotes have three rRNA genes namely the 5S, 16S and the 23S, with the 5S being the smallest and the 23S being the largest. For conclusive and stable phylogenetic comparisons it is essential that the genetic material being compared is substantial in size. It was found that the information within the 5S RNA gene was not sufficient and resulted in erroneous phylogeny (Ludwig et al., 1998). The use of the 5S rRNA gene for phylogenetic comparisons waned in favor of the 16S rDNA gene. The gene carrying the most genetic information is the 23S rDNA gene, but because of its large size it is at present still too cumbersome to sequence in its entirety and fewer 23S rDNA sequences are deposited in the databases. Because of its larger genetic information the 23S rDNA gene sequence will have a greater resolving power for phylogenetic reconstruction than that of the 16S rDNA gene (Ludwig et al., 1998, Ludwig and Schleifer, 1999).

All the rRNA molecules share a common secondary and higher order structure but vary within their primary structure. There are always highly variable regions found within the primary structure. These regions can be utilized for differentiating strains and species. As mentioned earlier, very little lateral transfer and genetic recombination within the rRNA genes has occurred and this has resulted in highly comparable sequence similarities between closely related organisms. This in turn makes the use of 16S rDNA gene sequence comparisons unstable at the species level but very effective above. It has been proposed that a rDNA sequence similarity of at least 97% and above represents a species, as it would most likely have a total DNA: DNA relatedness of no less than 70% (Wayne et al., 1987). It is also important to remember that there is no linear correlation between overall DNA: DNA hybridization similarities and 16S rDNA gene similarities because of the highly conserved nature of the latter.

1.10.1.4 DNA fingerprinting/typing

This technique is used in understanding the intraspecific species diversity. It is not suitable for use in the circumscription of prokaryotic species. There are two classes of DNA fingerprinting techniques, namely the first generation typing methods and the polymerase chain reaction (PCR) based methods.

The first generation typing methods are based on digestion of the whole genome with restriction enzymes. Rare cutter enzymes that recognize 6-8 bp sequences are used in order to obtain large restriction fragments. These fragments are too large to be properly separated by conventional agarose gel electrophoresis and, therefore, pulse field gel electrophoresis (PFGE) has to be used (Vandamme et al., 1996, Maslow et al., 1993, Tenover et al., 1995). PFGE is normally used in order to estimate the size of the genome. PFGE is based on the principal of continuously changing the direction of the current that flows through the gel system. This slows the migration of the DNA fragments down considerably allowing separation of the large fragments, which re-align with the direction of travel more slowly than the smaller fragments. Banding patterns unique to strains within species are easily identified. When common restriction enzymes are used to create a vast variety of restriction fragments a technique called Southern hybridization can be used in order to gain information about the genome of the organism. Southern hybridization is commonly used to estimate the number and distribution of rDNA copies within the genome. This is known as ribotyping and is a useful feature when classifying strains within a species (Grimmont and Grimmont, 1991).

PCR based methods rely on amplifying a specific area of the genome creating a banding pattern unique to specific organisms. There are a plethora of PCR-based typing techniques currently being used to group organisms. Of the most commonly used is the random amplified polymorphic DNA (RAPD) technique where short random oligonucleotides are used as primers for the PCR reaction. Unique banding patterns make it possible to distinguish between different strains (Williams et al., 1990).

1.10.2 Phenotypic information

The phenotype of an organism is the observable expression of its genotype. Before molecular techniques were developed for prokaryotic classification, taxonomists relied solely on comparisons of the phenotypes of the studied organisms. This led to a biased classification that favored aerobic heterotrophic microorganisms for which extensive retrieval of information was available (Roselló-Mora and Amann, 2001). Another major disadvantage of analyzing the phenotype is that the whole information potential of the genome of the prokaryote is never expressed. This is mostly because of the activation of gene expression in response to certain environmental conditions.

The analysis of the phenotype of prokaryotes relies heavily on the development of techniques that test the different phenotypic properties of the microorganisms either directly or indirectly (e.g. enzyme activities, substrate utilization, etc). This is a result of the very limited complex morphological features and life cycles of the prokaryote. Unlike genomic information, phenotypic data is usually compared phenetically. This means that the comparison is made through a large set of independent co-varying characters (Sneath and Sokal, 1973, Sneath, 1989). The comparisons reflect the degree of similarities between the organisms under analysis.

Several problems arise when using phenotypic methods for analysis. The most important of these is the fact that different character sets may show very poor congruence. Another important problem as mentioned earlier is that the phenotype only represents a very small part of the prokaryotic genome (Logan, 1994). These problems are addressed by basing the classification on as large a number of characters from as wide a phenotypic range as possible. There are several methods used in phenotypic analysis of prokaryotes.

1.10.2.1 Classical phenotypic analysis

Classical phenotypic tests are used in identification schemes in the majority of microbiological laboratories. While genomic data is sufficient to allocate taxa on a phylogenetic tree and is helpful in drawing major borderlines in the classification system, consistency of phenotypic and genomic characters is required to generate a useful classification system and may influence the depth of the hierarchical line (Wayne et al., 1987). The classical phenotypic character of bacteria consists of morphological, physiological and biochemical features. The morphology of a bacterium includes cellular (i.e. shape, endospores, flagella, Gram staining, etc.) and colonial (color, form, dimensions, etc.) characters. The physiological features include data on properties such as growth at different temperatures and pH values, salt concentration or atmospheric condition. Biochemical features of bacteria include information such as the presence or activity of various enzymes and metabolism of various compounds, etc. A major problem with classical phenotypic analysis is the lack of reproducibility of the experiments (Wayne et al., 1987). This may be overcome by utilizing highly standardized procedures.

1.10.2.2 Numerical (phenetic) analysis

Numerical evaluation can be described as the grouping by numerical methods of taxonomic units into taxa on the basis of their character traits (Sneath and Sokal, 1973). This method, as mentioned earlier, involves the conversion of information on taxonomic entities into numerical quantities. Phenetic analysis is often regarded as a synonym for numerical analysis and involves 5 important steps: (i) Selection of the strains (using as large a number as possible), (ii) selection of the type of tests (100 – 200 tests being optimal), (iii) encoding the data (positive response as 1 and a negative response as 0), (iv) computer analysis resulting in the generation of dendograms and (v) the presentation and interpretation of the results. The selection of a large number of tests for obligate autotrophs is much more difficult as these organisms have a very limited physiology. Numerical taxonomy has supported the development of a stable prokaryotic classification system, especially with the determination of homogeneous groups that can be equated with species.

1.10.2.3 Chemotaxonomy

Because phenotypic methods comprise all methods that do not include analysis of DNA or RNA, chemotaxonomic methods have to be included as well. This method refers to the analysis of on the chemical constituents of the prokaryotic cell. It is important to ensure that the variation in the chemical composition of the organism is due to genetic differences and not because of a change in the cultivation conditions. Several techniques such as the determination of the cell wall composition and the ratio and composition of polyamines and lipids are increasingly being used.

1.10.2.4 Phenotype typing methods

As with the genomic typing methods, the phenotypic typing techniques (i.e. serotyping, protein electrophoresis profiles, etc) are very useful in establishing relationships within bacterial species, but normally lack resolving power above the species level (Roselló et al., 1992, Roselló-Mora et al., 1994, Vandamme et al., 1998). Several techniques have already been established that are able to discriminate between strains and help with the understanding of intraspecific variability

1.10.2.5 Identification keys and diagnostic tables

Identification keys make use of a framework in which the identity of the isolate is tested using an orderly, step-by-step series of questions. Diagnostic tables, which are more commonly used in microbiology, contain considerably more information than the identification keys (Trüper and Schleifer, 1992). Diagnostic tables are based on the sharing of several unweighted characters, which are characteristic to, and will help identify, the taxon. The tables also include variable characters, which give a insight into the intraspecific diversity of the taxa. In order to give an accurate description of a species, a minimum of 10 – 25 strains should be studied. In the majority of cases, however, new species and genera are often described on the basis of only a few strains (sometimes only a single strain). Poor description of species based on small sets of strains can result in the incorrect circumscription of new taxa, which in turn will hinder the correct classification of new isolates (Roselló-Mora and Amann, 2001).

1.10.2.6 Automated identification systems

These are miniaturized phenotypic fingerprinting systems, which are based on classical phenotypic methods (e.g. API from Analtab Products, Plainview, NY, USA,) (D'Amato et al., 1991). Although commonly used, some taxonomists feel that caution should be taken when using these identification systems because of the reduced set of tests they utilize (Palleroni, 1997). Recently more advanced non-commercial systems have been developed which include over 200 different tests. These systems have proven to be very successful in the examination of the physiological diversity of environmental samples (Roselló-Mora et al., 1994, Kämpfer et al., 1991) even though it is not suitable to all types of microorganisms.

1.11 Prokaryotic species concept

Presently there are more than 20 different species concepts under scrutiny for prokaryotic organisms. Of the 20 there are only two that can be universally applied to all living organisms (Mayden, 1997; Hull, 1997).

1.11.1 PhSC (phenetic or polythetic species concept)

This concept is based on statistically co-varying factors, which are not necessarily universal among the members of a taxa (Hull, 1997). The PhSC system has proven to be stable and operational in the classification of the prokaryotic species and covers most of the requirements for a species, namely universality, monism and applicability. This concept has no theoretical commitment.

1.11.2 ESC (evolutionary species concept)

This species concept is the most theoretically committed of all the studied concepts (Mayden, 1997; Hull, 1997). It is a concept that is based on lineage but has no pragmatic significance for prokaryotes as their evolutionary fate and historical tendencies cannot be recognized because of lack of fossil records. Horizontal gene transfer makes the prediction of evolutionary tempo and changes even more difficult.

Another concept has been brought forward recently even though it is not universally applicable.

1.11.3 PSC (polygenetic species concept)

This concept consists of two versions, both of which are based on phylogeny or genealogies. The monophyletic (autapomorphic) species concept states that a species is the least inclusive monophyletic group definable by at least one autapomorphy (Hull, 1997). This can only be done if the nucleotide sequence of a particular gene is known that is exclusive to all members and that is homologously unique. This gene also needs to be excluded from the possibility of undergoing horizontal gene transfer. This is nearly impossible and thus this concept is not operational for the prokaryotes. The second concept, namely the diagnostic species concept defines a species as the smallest diagnosable cluster of individual organisms where there is a parental pattern of ancestry and descent (Hull, 1997). The strict application of this concept can lead to the simplification of the unit, which contrasts with observations that the prokaryotic species cannot be regarded as a smallest diagnosable unit (Vandamme et al., 1996; Ursing et al., 1995).

The prokaryotic species concept can be referred to as a phylo-phenetic species concept, indicating a combination of phenetic evaluation of the unit with requirements for monophylism of its components. A phylo-phenetic species can be described as a '*monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property.*' (Roselló-Mora and Amann, 2001)

1.12 Project aims

Mineral biooxidation is a combination of chemistry and biology. The actual leaching reactions are chemical, the biological component (microorganisms) being responsible for the regeneration of the chemicals. The susceptibility of different minerals to chemical attack varies substantially. One of the ways in which to improve the biooxidation rates of difficult to decompose mineral sulfides is to operate the biooxidation processes at elevated temperatures. Mineral oxidation is an exothermic reaction, therefore the temperatures within the biooxidation tanks and heaps tends to increase. In order to maintain a temperature within the tanks in which the microorganisms can flourish, extensive cooling systems need to be in place. These cooling systems increase the energy requirement of the biooxidation plants and have a negative economic impact on the biomining process. The optimium temperature for mineral biooxidation therefore varies from mineral to mineral depending on the reaction rate and amount of cooling required. A need within the mining community has thus been identified for a suite of biooxidation processes that operate at ambient to 35°C, 35°C - 45°C, 45°C - 55°C, 60°C - 65°C and greater than 75°C. It has been shown that Sulfobacilli are important members of the microbial population in processes that operate at 45°C - 55°C.

Bacteria that operate at 40°C and lower have been most studied. The present knowledge base, for *Sulfobacillus* spp. is rather limited however. The main aim of this project was to expand the available information regarding the biodiversity of these organisms. Thirteen strains of Sulfobacilli from areas around the globe were chosen for this study. An additional two strains from a related bacterium, *Alicyclobacillus* spp. were included for comparative purposes. Two types of taxonomic tools namely phenotypic and genomic analysis were used in order to accumulate as much information about Sulfobacilli as possible.

It is important to establish which *Sulfobacillus* species are present within the South African industrial biooxidation plants in order to identify those species that are worthy candidates for further investigation. Major research programs such as the development of genetic systems for the introduction of genes for increased metal resistance can then be economically undertaken for these important species.

A second aim of this study was to develop a method for identification of *Sulfobacillus* and its individual species. A pre-requisite of this technique was that ideally it had to use fairly low technology and be inexpensive and rapid.

CHAPTER TWO

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2.1 <u>Introduction</u>

This chapter deals with the phenotypic analysis of the genus *Sulfobacillus*. Although phenotypic data is not sufficient for the delineation of bacteria into separate species, it has proven to be a helpful tool for showing the degree of intra- and infra specific similarity between the species under analysis. Furthermore, it help to expand the information on the metabolic capabilities of these bacteria.

A classical phenotypic analysis approach was undertaken during the study of *Sulfobacillus* and related organisms (See Chapter 1, section 2.1.2.1). The morphological features studied include the cellular and colonial characteristics. Physiological features studied included optimal growth determination at different pH and temperature values as well as the strains ability to respire anaerobically. Biochemical data was obtained by testing the degree of utilization of various compounds.

2.2 Material and Methods

2.2.1. Bacterial strains

Thirteen strains of *Sulfobacillus*, isolated from a large geographical area were used for intra and infra specific comparisons (Table 2.1). A further two strains from the genus *Alicyclobacillus* were incorporated in this study. This was done in order to draw a comparison between the two different genera.

Table 2.1. Strains and sources of *Sulfobacillus* and *Alicyclobacillus*

Species/Strains	Source	GenBank Accession number of 16S rDNA sequence
S. thermosulfidooxidans		
611†	Commercial bioleaching operation (SA)	-
Adapt†	Commercial bioleaching operation (SA)	-
Kara* (DSM9293)	Russia	X91080
MT13*†	Commercial bioleaching operation	-
S. acidophilus		
TH1*†	Iceland (Thermal spring)	-
ALV/NAL*	England (Coal spoil)	AF050169
S. yellowstonensis		
YTF1*	Yellowstone National Park (Thermal spring)	AY007665
YTF5*†	Yellowstone National Park (Thermal spring)	-
GG6/2*†	Montserrat Island	-
S. ambivalens		
Riv-14*	Montserrat Island	AY007664
GG6/3*†	Montserrat Island	-
THWX*†	Whales (Coal spoil)	-
S. montserratensis		
L-15*	Montserrat Island	AY007663
Alicyclobacillus		
SLC 66*	Salt Lake City, Uttah	AY040739
GSM*	Nevada	AY007662

*Strains kindly supplied by D.B. Johnson; † Species identification done within this study.

2.2.2. Culture media and growth conditions

All strains were routinely grown in 800 ml ferrous sulfate media (% w/v): $(NH_4)_2SO_4$ 1.0; $MgSO_4$ 0.4 supplemented with tryptic soy broth 0.2, 500 mM $K_2S_4O_6$ (1ml/200ml), 500 mM $FeSO_4.7H_2O$ (1 ml/20 ml) and 1000X trace elements solution (1 µl/10 ml). Culture pH was adjusted to pH 2.5 with concentrated H_2SO_4. Cultures were incubated in flat-bottomed flask at 40°C with shaking. When testing for the presence of endospores, cells were grown using a poor energy source. In these experiments, yeast extract (YE)-media (($(NH_4)_2SO_4$ 1.0; $MgSO_4$ 0.4, YE 0.4 and supplemented with 500µM $FeSO_4.7H_2O$) was inoculated with 10% v/v actively growing cells taken from ferrous sulfate media. The cultures were incubated at 40°C for 7 days after which spectrophotometric readings were taken. Media used throughout this study is described in full detail in Appendix A.

2.2.3 Ferric iron reduction

A physiological feature of some Sulfobacilli strains is their ability to respire anaerobically. The ability of *Sulfobacillus* and *Alicyclobacillus* to respire anaerobically utilizing ferric iron as the final electron acceptor was tested.

Ferric iron media (25 mM ferric sulphate,10 mM glycerol, 1x basal salts, 0.02% YE) was prepared and the pH adjusted to between pH 2.0-2.5 with concentrated H_2SO_4 prior to being autoclaved. The autoclaved media was cooled to approximately 50°C before it was mixed with cooled, autoclaved 1.2% agarose media. Plates were poured and left to dry at 37°C for approximately 1 hour. Prior to inoculation, the plates were stored in an anaerobic chamber for one day in order to render them completely anaerobic. A threefold dilution of an actively growing culture was prepared and spotted onto the ferric iron plates within the anaerobic chamber. The plates were subsequently incubated in the anaerobic incubator at 45°C for 10 days. It is important to note that without a humidifier or water source within the incubator rapid drying out of the plates took place and no growth was possible. The presence of clear halos surrounding cell growth within the ferric iron plates indicated the ability of the strain to convert ferric iron (red/orange) to ferrous iron (colorless).

2.2.4 **Colony morphology**

One of the few visible morphological characters that can be utilized in order to distinguish between organisms is the size, shape and texture of their colonies when grown on solid media. For this purpose SJH-potassium tetrathionate overlay plates were prepared as described by D.B. Johnson (1995). Actively growing cells were plated using the spread plate technique and the overlay plates incubated at 37°C until growth was visible. . The resulting colonies were studied microscopically at 100 times magnification. Photographs of the overlay plates were obtained using a Nikon FDC-35 camera mounted on a Nikon eclipse E400 microscope. The plates were returned to the 37°C incubator and studied over an additional 10 days in order to determine the changes that occured during colony aging. Besides the determination of the typical colony morphology for both *Sulfobacillus* and *Alicyclobacillus*, this technique was used to ensure culture purity from time to time.

2.2.5 Mixotrophic growth

It has been noted previously that some of the strains were able to sustain growth by utilizing glycerol instead of yeast extract or trytic soy broth. Assortments of sugars (Table 2.2) were chosen in order to test the ability of the strains under investigation to grow mixotrophically. Actively growing cells from ferrous sulfate media supplemented with glycerol were harvested by centrifugation (10000 rpm), washed in pH 1.7 acid water and resuspended in pH 2.0 basal salts. The bacteria were inoculated (2% v/v) into 10 ml ferrous sulfate media supplemented with one of the sugars (5 mM final concentration). The pH was adjusted to 1.8 and the culture incubated in a rotating wheel at 37°C for 7 days.

The cells were harvested by means of centrifugation at 10 000 rpm, resuspended in 1 ml TE buffer and the optical density (OD) at 600nm determined spectrophotometrically. An uninoculated tube was used as a negative control to rule out erroneous readings due to the possible formation of ferric iron precipitate (jarosite) and contamination. A baseline growth capacity was determined by inoculating actively growing cells into ferrous sulfate media without any sugar supplementation. An OD(600nm) of 0.001 higher than the baseline value indicated a slight ability for mixotrophic growth, an OD(600nm) of 0.01 higher than the baseline value indicated a moderate ability for mixotrophy and an OD(600nm) of 0.1 higher than the baseline indicated a very good ability to grow mixotrhophically. It was presumed that the addition of the sugar had no effect on growth when there was a difference less than 0.001 above or below the baseline OD(600nm). Inhibition of growth by the substrate was indicated by a decrease of the OD(600nm) by more than 0.1 below the baseline OD(600nm).

Table 2.2 Sugars used to test for mixotrophic growth

Hexose (C6) sugars	
	Glucose
	Fructose
	Mannose
	Galactose
	Glucosamine
	Glucoronic acid
	Galactoronic acid
Pentose (C5) sugars	
	Arabinose
	Xylose

2.2.6 **Cell morphology**

Another visible phenotypic characteristic used to distinguish between organisms is the cell shape, presence of endospores and their cell wall composition as determined by Gram staining. Cells were grown and harvested as described in 2.2.2. Gram stains were carried out on all strains These experiments were repeated in order to ensure correct typing. It was found that a Gram variable result was obtained for some of the organisms. For these organisms' two more successive Gram stains were obtained each time making use of newly prepared cells.

Endospore formation was encouraged by growing the cells in energy poor media containing yeast extract (YE), mineral salts and very small amounts of ferrous iron for several days as described in section 2.2.2. Spore staining was done and the presence or absence of endospores visualized using a microscope.

It has been noted by previous researchers that *Sulfobacillus* has the ability to form a wide variety of cell shapes during different modes of growth. Cells were grown mixotrophically with YE or glucose, heterotrophically with YE and autotrophically with ferrous sulfur as its sole energy source. Cells were harvested by centrifugation at 10000 rpm, heat fixed in duplicate on microscope slides and stained with safranin. Cells were viewed under the 1000x magnification as either stained or unstained cells.

2.2.7 **Optimal pH range**

YE media were prepared with pH values ranging from 1.0 to 6.0. No ferrous sulfate was added, as this would precipitate out of solution at high pH values. Actively growing cells (10% v/v) were inoculated in 50 ml YE media and incubated for 5 days at 40°C with shaking. Cells were harvested using centrifugation at 10 000 rpm and resuspended in 1ml TE buffer. The optical density (OD) of the cultures was determined spectrophotometrically at 600nm.

2.2.8 **Optimal temperature range**

Actively growing cells were inoculated into 50 ml ferrous sulfate media supplemented with potassium tetrathionate (PTT). The pH was adjusted to 2.5 with concentrated H_2SO_4. Cultures were incubated with shaking at various temperatures for 5 days. Cells were harvested by centrifugation at 10 000rpm and resuspended in 1ml TE buffer. Their optical densities (OD) at 600nm were determined spectrophotometrically.

The culture purity was tested after determining the isolates ability for mixotrophic growth, optimal pH- and temperature ranges, using either a PCR technique (see Chapter three) or by means of spread plate techniques.

2.3 Results and discussion

2.3.1 Ferric iron reduction

The ability of *Sulfobacillus* and *Alicyclobacillus* strains to grow under anaerobic conditions was tested by spotting actively growing cells onto ferric iron plates. The result of this experiment is summarized in Table 2.3. Strain GG6/3 and MT13 were the only two strains that exhibited weak growth after the 20-day incubation period without the formation of a ferric iron reduction zone. It is likely that the growth of these strains was too weak to result in a visible halo being formed. Extended incubation would most likely have resulted in the presence of a cleared zone. The lack of growth on the ferric iron plates is not necessarily an indication that those strains are incapable of utilizing ferric iron during anaerobic growth. It is quite possible that the growth rates of these organisms are severely impaired by the lack of a suitable energy source and electron acceptor. This experiment was done in triplicate in order to verify the results.

It is known that some organisms can utilize ferric iron as an alternative electron sink for respiration purposes under anaerobic conditions. Acidophilic bacteria are especially suited for ferric iron respiration as ferric iron is present in its soluble form at the low pH were these organisms flourish. This respiration process produces enough energy in order to maintain cell growth. During the respiration process the ferric iron (Fe^{3+}) accepts an additional electron, thus being reduced to ferrous iron (Fe^{2+}). This reduction of ferric iron results in a color change from orange-red to cleared white zones. It is therefore possible to visibly identify organisms that have the capacity to reduce ferric iron under anaerobic conditions (Fig.2.1).

Inoculated plates were incubated for 10 days. During this period the humidity within the incubator was maintained by keeping a water reservoir full. The plates on which growth was present were removed from the incubator and photographed. The amount of ferric iron reduction and colony growth was quantified visibly by comparing the size of the halo formed. Those plates on which no ferric iron reduction halos were visible were left in the incubator for a further 10 days. The loss of moisture during the prolonged incubation inevitably led to the drying of the plates and it was difficult to maintain the plates for longer than 20 days.

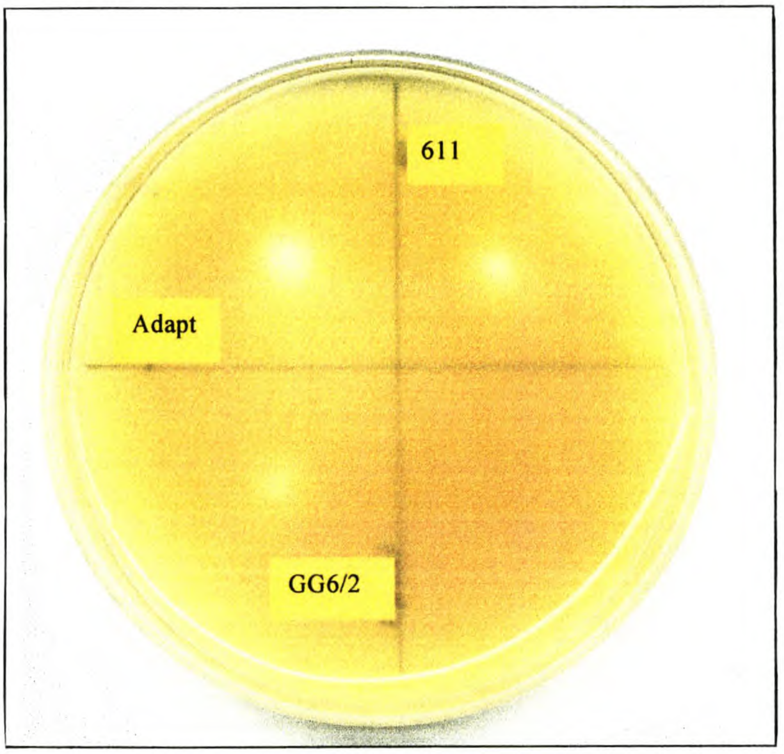

Figure 2.1 Photograph of clear halos as formed by ferric reduction on ferric iron reduction plates. The three different levels of ferric reduction can clearly be seen (slight iron reduction ✓; Average iron reduction ✓✓✓; Good iron reduction ✓✓✓)

2.3.1 Colony morphology

The SJH overlay technique is a well-established technique used in the isolation of pure cultures of these difficult-to-grow bacteria. All strains used in this study were plated out on overlay plates in order to confirm the maintenance of the pure cultures. Colonies of iron oxidizing bacteria are easily identified due to their characteristic red to orange hue. This typical color change is due to precipitation of ferric iron, which is formed after ferrous iron oxidation. A good example of a young colony can be seen in Figure 2.2.

Tabel 2.3 Ability to respire anaerobically utilizing ferric iron

Species/Strain	Ferric iron reduction
S. thermosulfidooxidans	
MT13	×
Kara	✓✓✓
Adapt	✓✓✓
611	✓✓
S. montserratensis	
L15	No growth
S. ambivalens	
Riv-14	✓✓
GG6/3	×
THWX	✓✓
S. acidophilus	
TH1	No growth
ALV/NAL	✓
S. yellowstonensis	
YTF1	No growth
YTF5	No growth
GG6/2	✓
Alicyclobacillus	
SLC66	No growth
GSM	No growth

✓ Slight ferric iron reduction; ✓✓ Average ferric iron reduction;
✓✓✓ Good ferric iron reduction; × No visible ferric iron reduction zone but definite growth visible

Initially, the colonies were very small and had a slight off-white color. They were smooth edged, slightly raised and had a dough-like consistency. With prolonged incubation at 37°C the ferric iron staining became more pronounced with the color changing from light orange to deep red (Fig.2.4 a-d). This change in color can be attributed to the increase in the concentration of ferric iron precipitate within the colony. It was also noted that the older colonies had a flatter appearance with a distinctive iron blush surrounding them compared to the younger colonies.

It is important to be able to distinguish *Sulfobacillus* from other iron oxidizing bacteria found within the biomining environment. To this effect *At. ferrooxidans* and *At. caldus* colonies were studied and compared to that of *Sulfobacillus*. A very distinctive iron blush surrounds the older colonies. A clear difference between

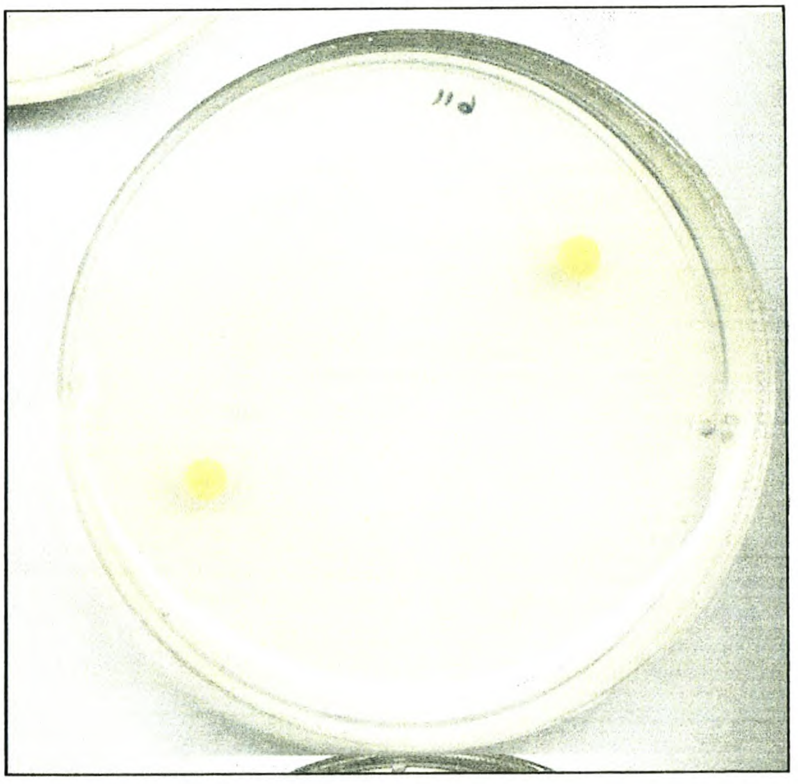

Figure 2.2 Typical colony after approximately 7 days incubation at 37°C

Sulfobacillus and *At. caldus* strains were visible as no orange to red color appeared with the latter bactera. A further difference is the dark black centers formed within the *At. caldus* colonies (Fig.2.3). These distinguishable characteristics were used to confirm the culture purity of *Sulfobacillus* strains from time to time

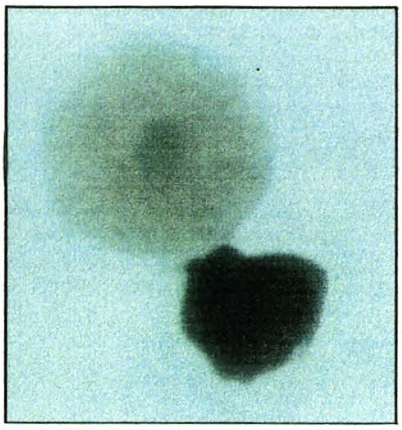

Figure 2.3. Comparison between *Sulfobacillus* colonies (top) and *At. caldus* colonies (bottom). Note the difference in size and colour of the centralized area.

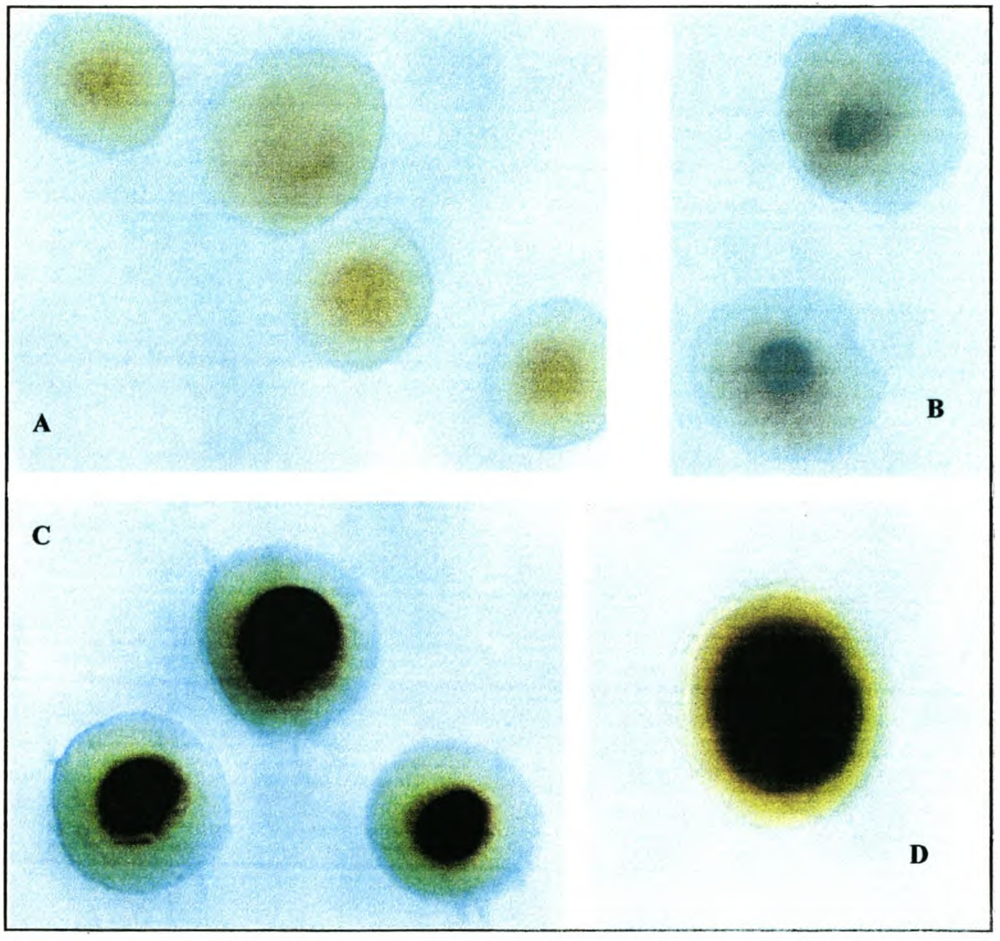

Figure 2.4 Microscopic photographs depicting the change over time of *S. acidophilus* (strain GG6/2) colony. All strains of Sulfobacillus tested display similar colony morphology. A: Colonies after 10 days, B: colonies after 13 days, C: colonies after 17 days, D: colony after 20 days.

2.3.3 Mixotrophic growth

The growth of Sulfobacilli strains in iron containing media supplemented with organic sugars was tested semi-quantitatively by comparing the culture optical density to that of the baseline cells to which organic supplements had not been added. The experiment was done in triplicate in order to allow for experimental variation. The growth of the different strains tested is shown in Tabel 2.4. The sugar utilization of *S. thermosulfidooxidans* strains Adapt, MT13 and Kara appear very comparable showing increased mixotrophic growth when galactose and glucosamine are added to the growth media. These strains form a tight phylogenetic cluster when comparing their 16S rDNA sequence (see Chapter 3). Strain 611 was also found to be a member of the species *S. thermosulfidooxidans*, but was slightly removed phylogenetically from the above-mentioned cluster. The sugar utilization pattern of this strain differs vastly from the other three members of *S. thermosulfidooxidans*. It appears as if strain 611 does not have the ability to grow mixotrophically using any of the sugars tested as no improvement in cell growth was observed.

The ability to utilize different carbon sources for *S. acidophilus* strains TH1 and ALV are very similar. It appears as if these strains have a higher affinity for pentose (C5) sugars than any of the other strains tested. The sugar utilization ability of strains from *S. yellowstonensis*, *S. ambivalens* and *S. montserratensis* varies considerably within and between species with no clear trend. Strains THWX and YTF1 both showed no responce, either positive or negative, to the addition of a sugar source to their growth media. It can be deduced that these strains do not have the ability to grow under mixotrophic conditions but are obligately autotrophic or se a carbon source other than those tested. Glucuronic acid is the only carbon source that was tested that did not have an enhancing effect on any of the strains tested. The presence of jarosite (iron precipitate) hindered the accurate estimation of cellular growth. It was found that under these conditions the growth of the organisms' was very limited. To overcome this problem the experiment was conducted in triplicate and an average of the cellular growth determined. Across the three experiments the results were highly comparable.

Table 2.4 Utilization of organic sugars by Sulfobacilli species

	Species												
Substrates	*S. thermosufidooxidans*				*S. acidophilus*		*S. m*	*S. ambivalens*			*S. yellowstonensis*		
	Adapt[1]	MT13[1]	Kara[1]	611[1]	TH1[2]	ALV[2]	L15[3]	Riv-14[4]	THWX[4]	GG6/3[4]	GG6/2[5]	YTF1[5]	YTF5[5]
Glucose	+	+	-	I	++	+++	-	+	-	-	-	-	-
Fructose	+	-	+++	-	-	-	+	I	-	-	++	-	I
Mannose	I	-	-	-	-	-	+	+	-	-	-	-	-
Galactose	++	+++	+++	-	-	-	+	-	-	-	-	-	I
Glucosamine	+	++	++	-	-	-	-	-	-	I	-	-	I
Glucuronic acid	-	-	-	-	-	-	-	-	-	-	I	-	-
Galactoronic acid	-	I	I	-	-	-	-	-	-	++	-	-	-
Arabinose	I	+	++	-	+	++	+	I	-	-	-	-	-
Xylose	-	-	-	I	+++	+++	+	I	-	-	I	-	+

S. m : *S. montserratensis*

\+ : culture OD > 0.001 above baseline OD;
++ : culture OD > 0.01 above baseline OD;
++ : culture OD > 0.1 above baseline OD;
\- : difference between culture and baseline OD of less than 0.001
I : Inhibitory, culture OD more than 0.1 below the baseline OD

2.3.4 Cellular morphology, stain and motlity

The majority of the strains tested stained Gram positive. For two of the strains (ALV and GG6/3) the results of the Gram stains were variable amongst different experiments indicating a change within the composition of the cell envelope. The strains were all incubated and tested simultaneously thus eliminating the effect that different growth conditions could have on the cell wall composition. A possible explanation for the difference in the Gram stain results could be that the Gram-variable strains were at different growth phases when compared to the original culture tested. These isolates nevertheless are probably all Gram positive as they group together with the other Gram positive Sulfobacilli.

The presence of endospores was determined by means of microscopic investigation prior and subsequent to endospore staining. No green endospores (typical of *Bacillus subtilis*) were obtained in any of the staining experiments even though endospores were clearly visible microscopically under the 1000-fold magnification. All strains tested had the ability to produces spores. The spores of both *Sulfobacillus* and *Alicyclobacillus* species were situated terminally and were normally only present at one end of the cell.

When cells were grown in ferrous iron media supplemented with tryptic soy broth, cells were present either individually or as long chains of up to 10 cells (Fig.2.5 A&B). Sulfobacilli has been shown to have different cell morphologies such as straight rods, coccoid, clavate (club-shaped) and pyriform (pear-shaped). The different morphologies and cell sizes of six strains are depicted in Figure 2.5. Some strains also produced cells that appeared branched when grown heterotrophically in the presence of glucose (Fig. 2.5 E). It was shown that strain YTF5 produced very elongated, distorted cells when grown in YE media supplemented with 5mM ferrous iron (Fig .2.5 C). This phenomenon has been noted by Norris (1996) for *S. acidophilus* strain ALV (included in this study but not shown), a close relation of *S. yellowstonensis* to which strain YTF5 belongs. All the strains tested showed limited motility (no flagella was observed) when grown either heterotrophically, autotrophically or mixotrophically.

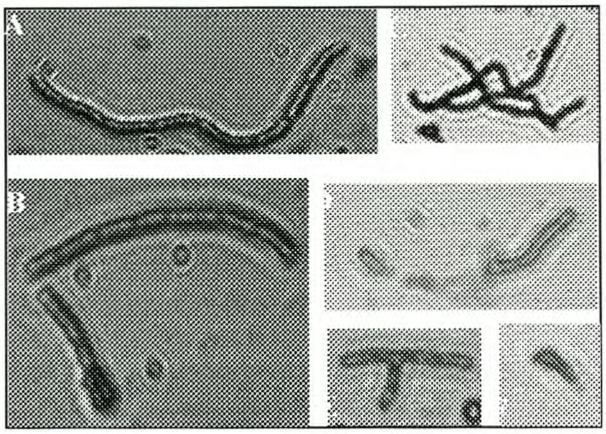

Figure 2.5 A & B: Chains of *Sulfobacilli* from strains 611(A) and Riv-14(B); C: Elongated cells from strain YTF5 grown hetrotrophically; D: Different cell sizes of strain GG6/2 during hetertrophic growth; E: branched cell formation of strain Kara growing mixotrophically in the presence of glucose; F: Clavate-(club) shaped cell of strain Kara.

2.3.5 Optimal pH range

The determination of the optimal pH range for growth of organisms is a commonly employed physiological characteristic that helps with the placement of the microorganisms within defined groups. In order for the biooxidation processes to be optimized correctly, sufficient data concerning the optimal pH ranges of the organisms involved, needs to be presented.

The optimal pH for both Group 1 and Group 2 Sulfobacilli (see Chapter 3 for 16S rDNA based grouping) is 2,5 (Fig.2.6 A & B). Of Group 1 Sulfobacilli only strain Riv-14 has a pH optimum different to that of the other strains tested. The optimal pH for strain Riv-14 is pH 2.0. Strains YTF5 and YTF1 belonging to *S. yellowstonensis*, together with Riv-14 were the only strains that were capable of good growth below pH 2.0. YTF5 shows as good growth at pH 2.0 as it does at pH 2.5. All strains bar Riv-14, YTF5 and YTF1 show very little growth below pH 2.0. All of the strains of *Sulfobacillus* showed negligible growth at pH 1.0. The range of pH values where growth was sustainable lies between pH 1.5 and pH 5.0. At pH 5.0 a white precipitate was formed after prolonged incubation. However, the precipitate settled to the bottom of the flask whereas the cells stayed in suspension. We can thus surmise that the precipitate had very little effect on the density readings at OD 600nm except that one is not certain whether nutrients (or what nutrients) were incorporated in the precipitate.

In has previously been shown (Norris 1996) that the species *S. acidophilus* had an optimal pH at approximately pH 2.0. This is slightly lower but similar to the optimal pH for the strains belonging to *S. acidophilus* (ALV and TH1) found in this study. Although their optimum pH was 2.5, the most acid tolerant isolates found in this study were *S. yellowstonensis* strains YTF1 and YTF5. Data obtained about the pH range for *S. thermosulfidooxidans* and related species compare very well with previously published data (Golovacheva, 1978) which states that limited growth was observed below pH 1.9 and that growth weakened above pH 3.0.

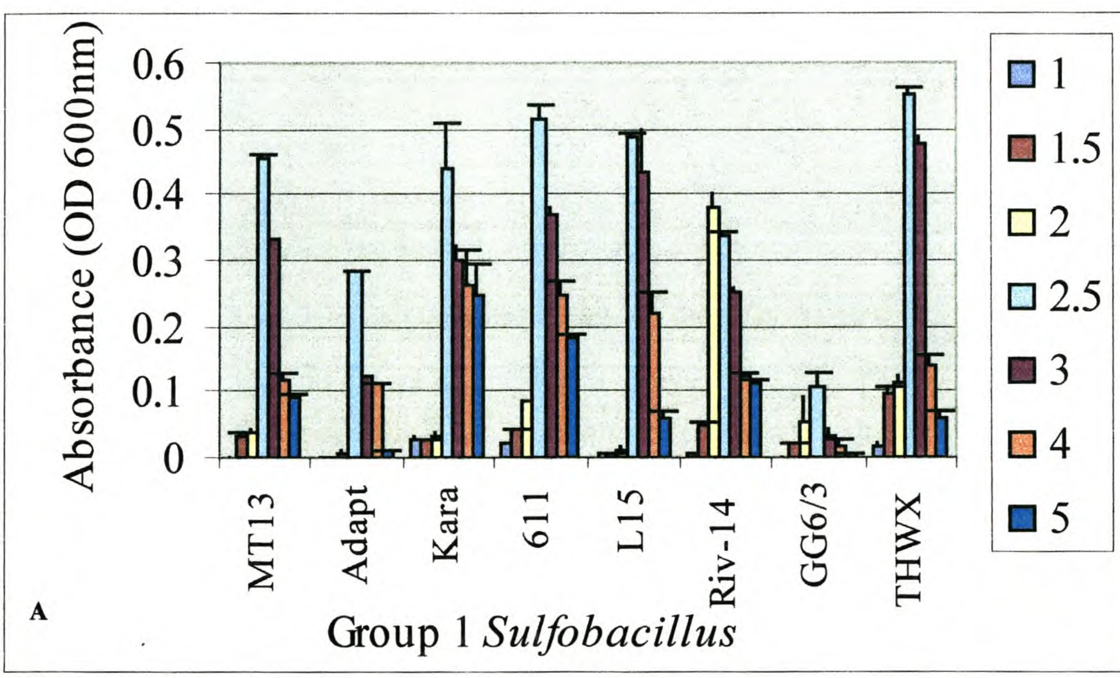

Figure 2.6 A The optimal growth range of Group 1 *Sulfobacillus*

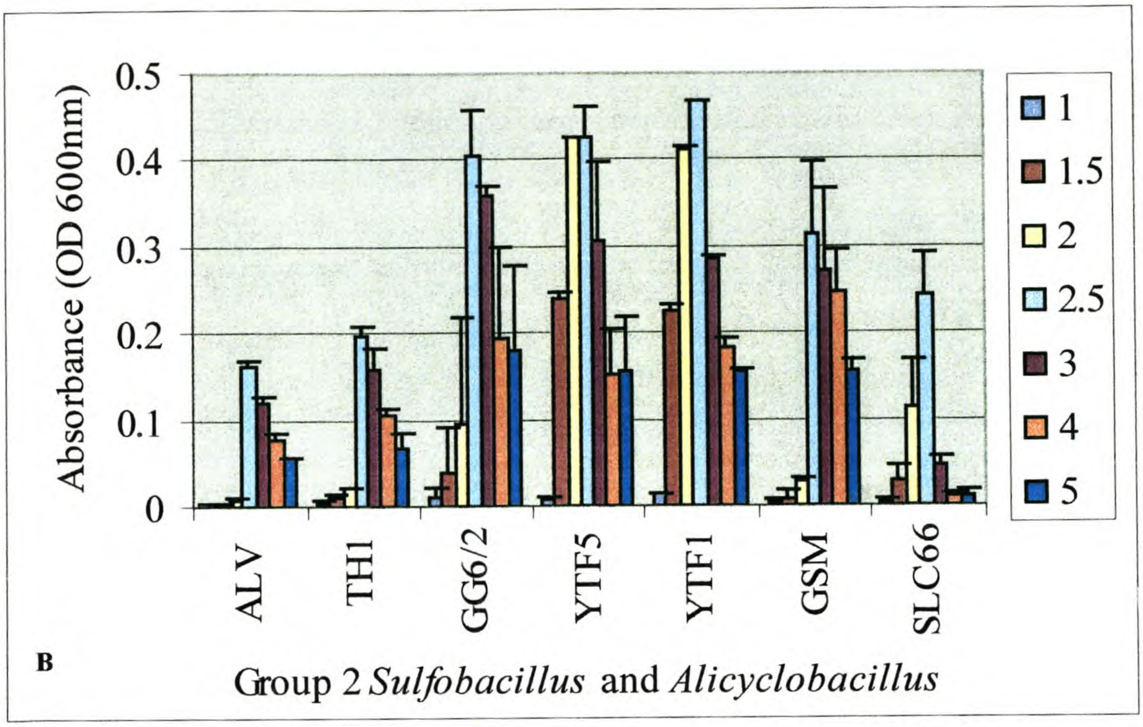

Figure 2.6 B The optimal growth range of Group 2 *Sulfobacillus* and *Alicyclobacillus*.

2.3.1 Optimal temperature range

It is essential to determine the optimal temperature range for the Sulfobacilli, as they are likely to play an important role within higher temperature biooxidation tanks.

Two distinct optimal temperatures were visible within strains of Group 1 Sulfobacilli (Fig.2.7 A). The cluster containing strains belonging to the species *S. thermosulfidooxidans* (strains Adapt, MT13, Kara and 611; see Chapter 3) exhibited optimal growth at 55°C. The rest of the strains of Group 1 Sulfobacilli grew optimally at a slightly lower temperature (45°C). All the strains except for strains L15 and Riv-14, showed very little growth at temperatures below 40°C. The growth of strains L15 and Riv-14 at 30°C - 45°C was much higher than any of the other strains, indicating that these strains have a higher affinity for growth at the lowest temperatures. Very little growth was noted for any of the strains at temperatures above 55°C. This correlates well with literature (Golovacheva, 1978) stating that the temperature maximum is around 60°C.

Organisms belonging to Group 2 Sulfobacilli (except strain YTF5) and *Alicyclobacillus* all had an optimal growth temperature of 55°C. Strain YTF5 belonging to the species *S. yellowstonensis* showed a temperature optima similar to that of strains L15, Riv-14, THWX and GG6/3 at 45°C. The temperature range of strain YTF5 however, differs from strains L15 and Riv-14 with proportionately little growth at 35°C or below, but high levels of growth at 50°C and 55°C. Norris (1996) noted that the optimal growth temperature for *S. acidophilus* (ALV and TH1) lies between 45°C – 50°C whereas in this study these bacteria grew best at 55°C.

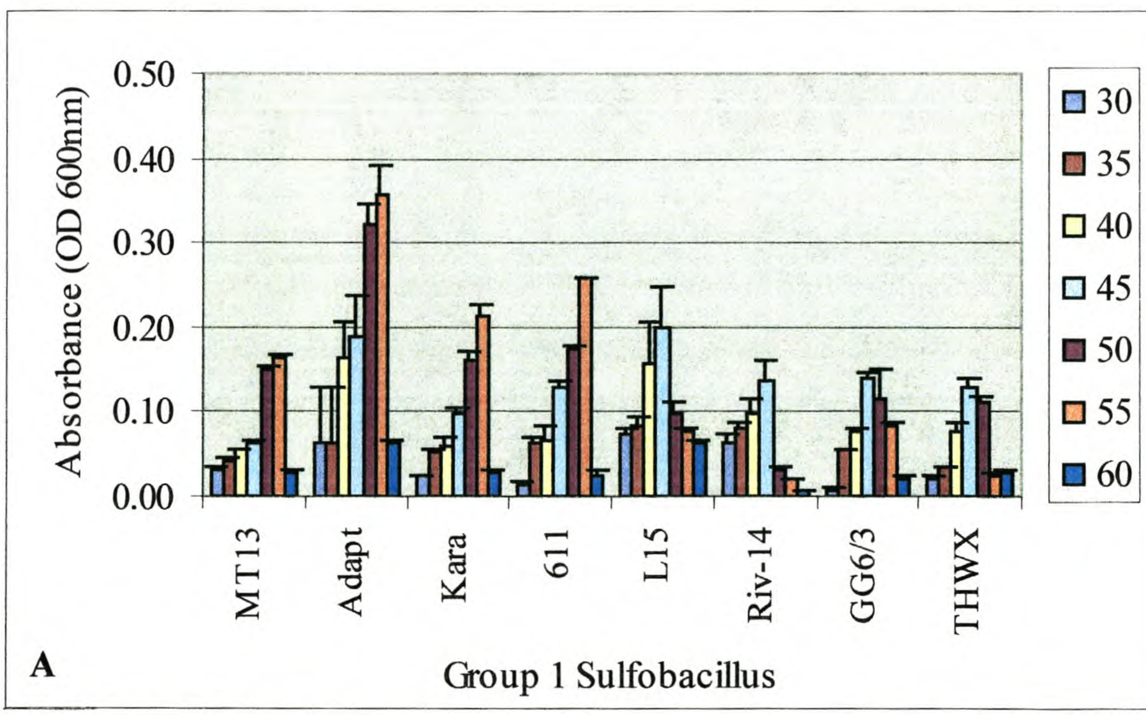

Figure 2.7 A The optimal temperature range for Group 1 Sulfobacilli.

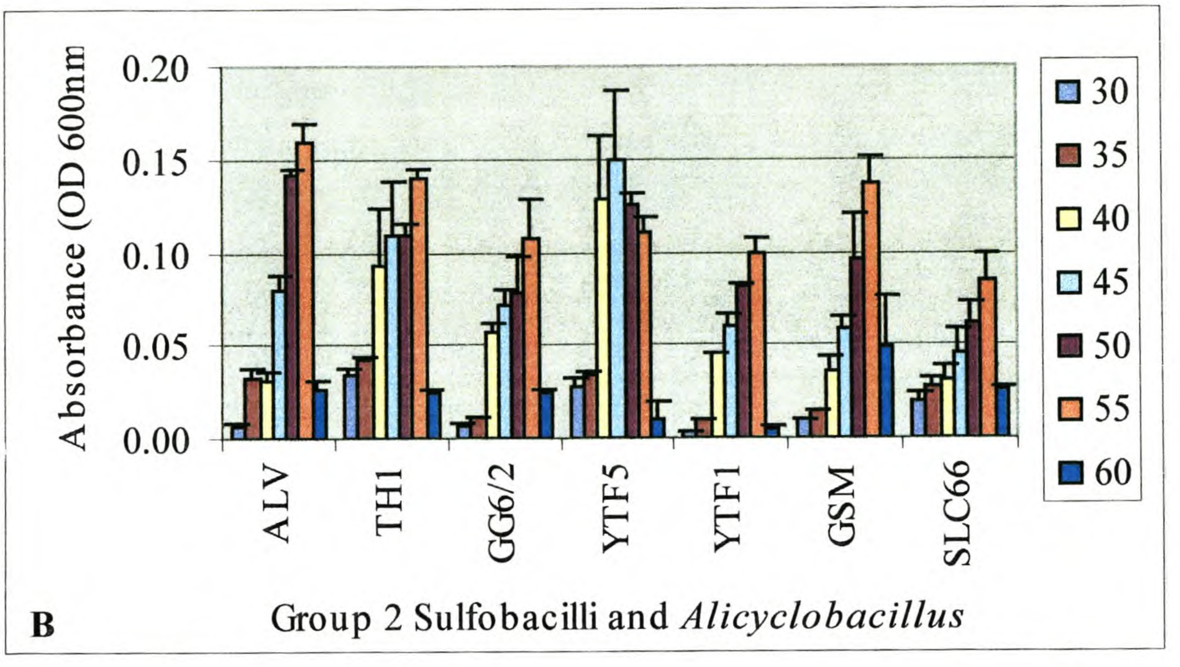

Figure 2.7 B The optimal temperature range for Group 2 Sulfobacilli and *Alicyclobacillus*.

Cultures incubated above 55°C formed a precipitate within 3 days presumably due to the chemical oxidation of ferrous iron. This in conjunction with the limited growth exhibited by all the strains made the accurate estimation of the optimal temperature range difficult.

As mentioned earlier (Chapter 1) Kovalenko (1982) reported the formation of distorted, filamentous cells at temperatures above 50°C for the *species S. thermosulfidooxidans* subspecies *thermotolerance*. These organisms grow optimally at temperatures around 42°C where their shape was undistorted. We tested whether any of the strains identified as belonging to *S. thermosulfidooxidans* and related species produced distorted cells above 50°C. We did not observe this phenomenon associated with *S.th.* subspecies *thermotolerance* for any of the strains.

The filamentous cells formation of *S.th.* subspecies *thermotolerance* only occur at temperatures above 50°C. The formation of filamentous cells by *S. yellowstonenesis* (YTF5) discussed in section 2.3.4 occurred at 45°C and is attributed to energy sources available rather than temperature optima.

CHAPTER THREE

Genomic analysis of *Sulfobacillus* and *Alicyclobacillus*

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3.1 Introduction

The current approach employed in order to classify organisms is known as the polyphasic classification system (Chapter one). In this system as many as possible phenotypic and genomic characteristics of the organism must be studied and compared. The phenotyipic analysis of *Sulfobacillus* and *Alicyclobacillus* is discussed in Chapter two.

The genomic characteristics of an organism can be divided into two groups namely those that can be deduced directly, such as the direct sequencing of certain genes (also known as phylogenetic analysis) and the genomic information that is gather indirectly such as DNA-DNA hybridization similarities; mol % G+C values.

1.1 Phylogenetic analysis

When choosing an area of the genome to study it is important to keep certain aspects in mind, for instance that all sequences are not of equal value in determination of phylogenetic relationships. According to C.R. Woese (1987) three important criteria has to be met before the specific sequence of a molecule can be credited as useful. The first states that changes in the sequence of the molecule have to occur as randomly as possible. The second factor has to take the rate of the changes into account. These rates have to be comparable with the spectrum of evolutionary distance being measured. The last criterion takes the size of the molecule into account, stating that the molecule must be of a sufficient size in order to provide a substantial amount of information. The 16S rDNA gene meets all these criteria, thus making it a very good candidate for use in determination of phlyogenetic relationships. As mentioned in Chapter 1, closely related organisms are often found to have a nearly identical 16S rRNA sequence. This makes species identification difficult as a point is reached where insufficient differences between the base sequences exist.

Ribosomal RNA plays a crucial role in all living organisms as it forms the core of the ribosome and its structure is important for protein translation. All 16S rRNA molecules appear to be nearly identical in function and their three-dimensional structure show only slight variation. It has been found that within this highly constant structure, sequence changes occur continuously within certain regions of the molecule. These changes however only result in alterations to the primary structure leaving a homologous secondary and tertiary structure. It is this large amount of neutral changes that allows the comparative analysis of the 16S rRNA of a variety of organisms (Guttel *et al.*, 1994). Eight hyper-variable regions are present in prokaryotic 16S rRNA (Neefs *et al.*, 1990). These variable regions are illustrated in Figure 3.2 based on the 16S rRNA secondary structure model of *Escherichia coli* (Brosius *et al*, 1978 and 1981)

The gene organisation of the ribosomal operon (*rrn* operon) of most bacteria can be seen in Figure 3.1 containing the following components: 16S rRNA, spacer, tRNA, spacer, 23S rRNA, spacer and 5S rRNA sequences (Watson *et al.*, 1987). There are several exceptions like *Borrelia burgdorferi* which has 2 copies of the 23S-5S rRNA and only one copy of the 16S rRNA gene separated from the first 23S rRNA gene by both tRNA^{ile} and tRNA^{ala} genes (Ojaimi *et al.*, 1994). Often more than one *rrn* operon is present within an organism [seven copies in *E.coli* (Morgan et al., 1997), ten in *Bacillus subtilis* (Loughney et al., 1982), ten in *Clostridium perfringens* (Garnier et al., 1991) one in *Mycobacterium* sp. (Bercovier et al., 1986), and one to two in *Mycoplasma* sp. (Amikam et al., 1984)]. Gürtler (1999) postulates that the rearrangements that occur within some bacteria are because of recombination events that occur between the multiple rrn operons. There is often considerable variation in both the sequence as well as the length of the spacer regions of the different *rrn* operons between species and sometimes between *rrn* copies within a single bacterial isolate. These spacer variations can be used for bacterial identification (Jensen, 1993), typing (Gürtler 1993, Cartwright 1995) and evolutionary studies (Anton 1998, Gürtler 1999).

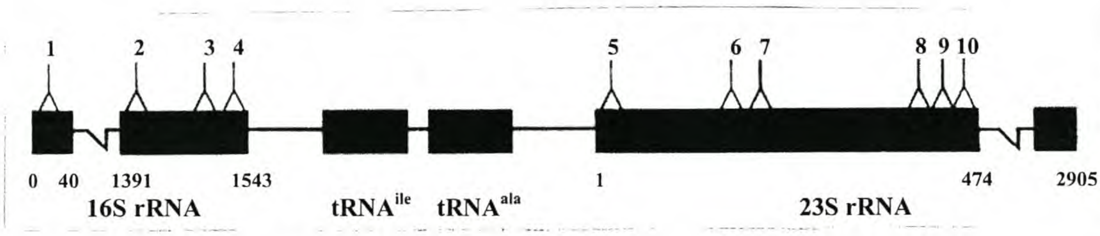

Figure 3.1 Conserved regions of within the ribosomal RNA (*rrn*) operon of *E. coli*. The boxed areas depict the various genes within the operon (the 5S rRNA gene not shown). Bold solid lines represent the intergenic spacer regions. Numbers 1-10 shows conserved regions with the following sequence blocks: (16S rRNA) **1**, 8-27; **2**, 1390-1407; **3**, 1491-1506; **4**, 1525-1542; (23S rRNA) **5**, 21-38; **6**, 115-132; 7, 188-208; **8**, 422-437; **9**, 441-460; **10**, 456-474 (Gurtler and Stanisich, 1996)

If a large enough group of sequences that flank the spacer regions is available for comparisons it is possible to design primers in order to amplify up the spacer regions. Regions 3 & 4 (Fig.3.1) are present in most eubacteria, region 2 is found in all domains (eubacteria, archaea and eukaryotes) and region 10 (within the 23S rRNA) is the best region for primer design. Next in order of conservation is regions 6, 9, 8, 7 and 5. Areas 2 and 10 are the regions of choice when it comes to primer design, with regions 2 and 7 also showing great success by various workers (Gürtler and Stanisich, 1996).

The sequence length variation is largely due to the number and type of tRNA molecules present. The majority of Gram-positive organisms have no tRNA genes within the rRNA region, and those that do, normally have either $tRNA^{ala}$ or $tRNA^{ile}$ or both. Gram-negative bacteria have both $tRNA^{ala}$ and $tRNA^{ile}$ or only $tRNA^{glu}$.

The evolutionary rate of change of the spacer regions have been shown to be much higher than that of the 16S rRNA gene sequence making the former useful in identification of recently diverged species (LeBonde-Bourget *et al.*, 1996). The 16S rRNA gene sequence sheds more light on the inter- and intragenic relationships of species and higher taxa (Fox *et al.*, 1992). Using the 16S rRNA sequences and the size of the 16S-23S rRNA spacer regions in combination increases the amount of information about the studied organism considerably.

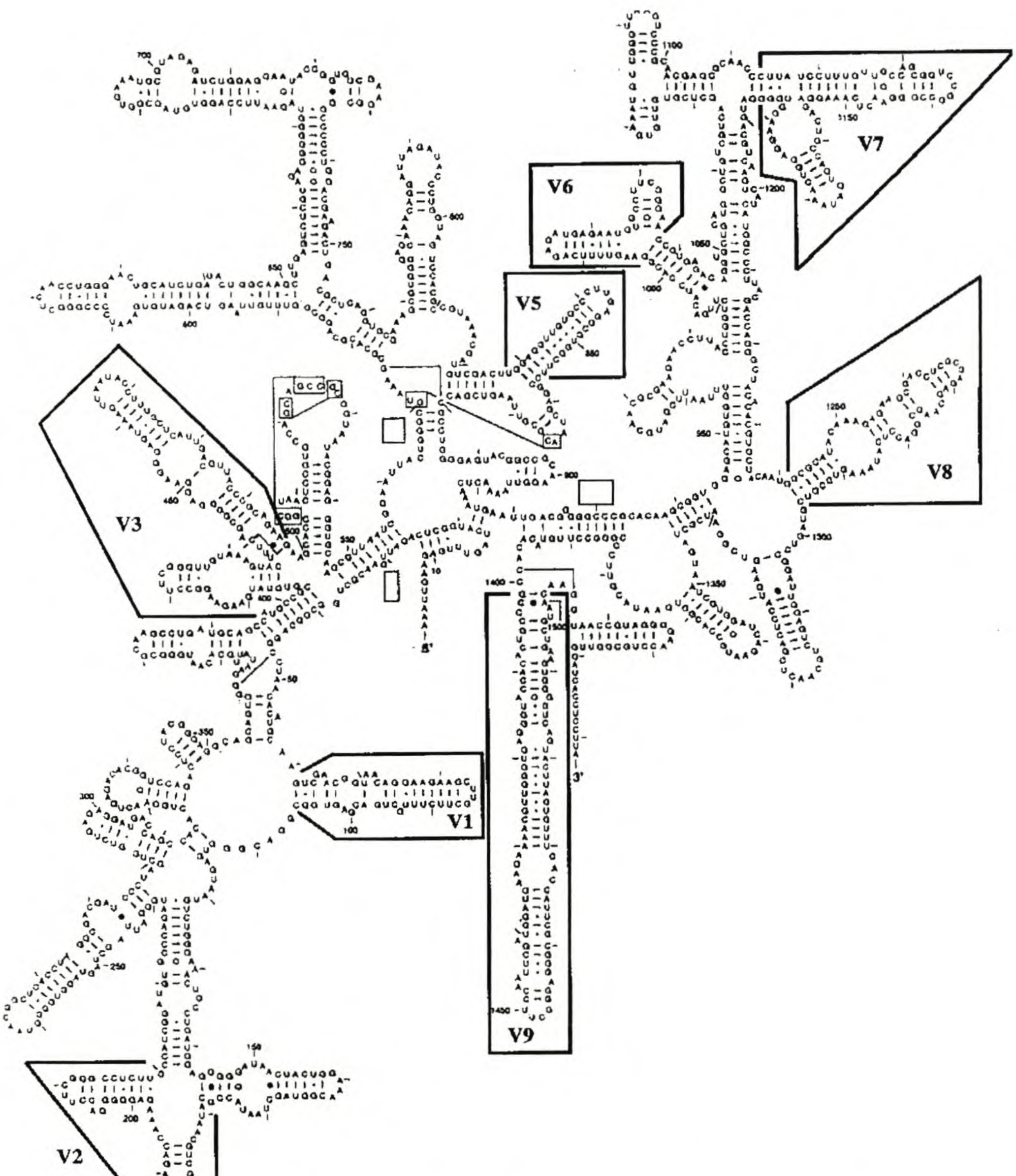

Figure 3.2 Secondary structure model for *E. coli* 16S rRNA displaying the hyper-variable regions numbered V1 - V9. V4 is not found in any prokaryotic ssRNAs. Canonical base pairs are connected by lines, G:U pairs are connected by dots, A:G pairs are connected by open circles and other noncanonical pairings are connected by solid circles. Thicker, longer solid lines connect "Tertiary" interaction. Every 10th position is marked and every 50th position numbered. The primary structure was determined by Brosius *et al*., 1978 and 1981.

1.2 Indirect genome analysis

The first method utilized to gather information indirectly about the genome of the organism was to determine the overall base composition of the organism (mol% G+C). This method is very crude in the sense that two organisms with the same or very close mol% G+C values do not necessarily belong to the same species as the linear sequence of the bases in the DNA molecule is taken into account. A more precise method namely DNA-DNA hybridization was developed. DNA-DNA hybridization takes the linear arrangement of the sequence into account, therefore making it a more accurate.

Due to the highly conserved nature of the 16S rRNA gene, there is frequently very little linear correlation between 16S rRNA sequence similarity and overall DNA-DNA similarity % (Stackenbrandt and Goebel, 1994; Grimont, 1988). The phylogenetic analysis of the 16S rRNA sequence is important in order to determine whether there exists sufficient relationship between organisms studied in order to invest in the more laborious DNA-DNA hybridization techniques. These methods are described in detail in Chapter one.

3.2 Material and Methods

3.1.1 Genomic DNA isolation

Genomic DNA (gDNA) was isolated from cultures grown at 45°C in ferrous sulfate media (Appendix A). Cells were pooled by centrifugation at 10 000 rpm for 30 minutes. The cell pellet was subjected to three consecutive acid water (pH 1.7) washes in order to eliminate as much of the ferric iron as possible. Successive cycles of low (2000rpm) and high speed (8000 rpm) centrifugation were done in order to purify the cell pellet further. The resulting cell pellet was resuspended in TE buffer and lysed by the addition of 10% sodium dodecyl sulfate (SDS). The lysate was incubated at 37°C with the addition of proteinase K (20mg/ml final concentration) until lysate became clear. Genomic DNA was extracted by addition of 100% ethanol and resuspended in TE buffer by incubating overnight at 37°C.

3.1.1 16S rRNA PCR amplification

Genomic DNA of approximately 100ng was subjected to PCR amplification. The reaction mix contained 20mM $(NH_4)_2SO_4$; 75 mM Tris-HCl (pH 8.8 at 25°C); 0.1% (v/v) Tween 20; 3 mM $MgCl_2$; 2.5 μM (each) deoxyribonucleotide (dATP, dCTP, dGTP and dTTp); 0.2 μM of each primer and 1U of Expand High-fidelity Taq polymerase (Roche biochemicals) for sequencing purposes or 1U of Taq polymerase (Promega) for restriction enzyme digestion purposes.

Three sets of standard primers (F1 5'-AGAGTTTGATCITGGCTCAG-3'; F3 5'-GCCAGCAGCCGCGGTAATAC-3'; F5 5'-GCATGGITGTCGTCAGCTCGTG-3'; R1 5'-GTATTACCGCGGCTGCTGGCAC-3'; R3 5'-CACGAGCTGACGACAICCATGC-3' and R5 5'-ACGGITACCTTGTTACGACTT-3'), designed from conserved regions, were used to for the amplification of the suitable fragment size of the 16S rDNA gene by PCR for sequencing purposes (see Figure 3.3). The Hybaid PCR Sprint temperature cycling system was used for this purpose. Two primers (fDD2 5'-

CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG-3' and rPP2 5'-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3') were used to amplify the entire 16S rRNA amplification for purposes other than sequencing. These 16S rRNA PCR products were subsequently cloned using the PGEM-T® vector systme (Promega).

The thermal profile involved an initial denaturing step at 94°C for 60 s, followed by 25 cycles of a 94°C denaturing step (30 s), an annealing step at 53°C for 30 s and an elongation step at 72°C for 90 s. A final elongation of 120 s was preformed at 72°C and a cooling step at 4°C for 60 s completed the reaction. Amplification products ranged from 418bp to 517 bp (fig. 3.2) in order to ensure coverage of the complete 16 S rDNA gene (1500bp) in both directions. The PCR products were purified using GFX™ PCR DNA and Gel Band purification Kit (Amersham Pharmacia Biotech Inc.) following the manufacturer's recommendations. The concentrations of the products were determined spectrophotometrically at 260nm.

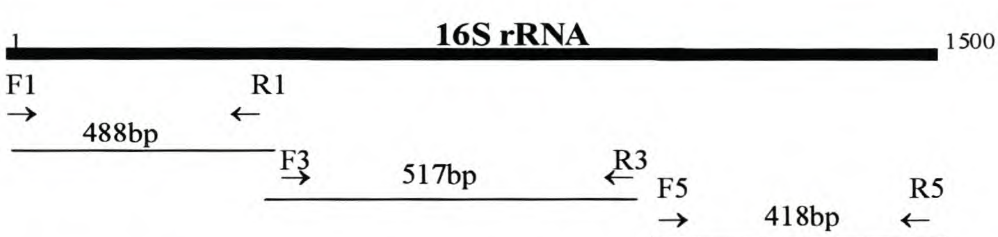

Figure 3.3 Schematic representation of the PCR primers used and estimated sizes produced.

3.1.3 **16S rRNA sequencing and analysis**

The sequencing reactions were preformed with the BigDye Terminator V3.0 Cycle Sequencing Kit (Amersham Parmacia Biotech UK Ltd.). A maximum of 40ng of template DNA (16S rRNA PCR amplification products) was used in the sequencing reactions. The reactions were run on an ABI PRISM® 3100 Genetic Analyzer. The sequence was processed with Sequencing Analysis V.3.7 Software. Template DNA was sequenced in both the forward and reverse reactions enabling full alignment of the complete sequence.

Phylogenetic trees and homology dendograms was done utilizing DNAMAN for Windows program, version 4.13 (1994-1999). Multiple sequence alignments were also obtained using the full optimal alignment option of DNAMAN.

3.2.4 16S –23S rRNA PCR amplification

Primers were designed using conserved sequence comparisons. The forward primer, rPP2flip (5'-AAGTCGTAACAAGGTAICCGTG-3') is based on the reverse primer (rPP2) used during 16S rRNA amplification and similar to primer G1 designed by Jensen et al (1993) for the specific amplification of the 16S-23S intergenic spacer region. The reverse primer, rev23 (5'-CGGTACTGGTTCACTATCGG-3') was designed using conserved areas further into the 23S rRNA gene. Primer rev23 lies approximately 400-500 bp into the 23S rRNA gene placing it within area number 10 as described by Gürtler and Stanisich (1996). This area has a much higher conservation than area 5 from where Jensen et al (1993) designed his reverse primer L1 that pairs with primer G1.

Amplification of the intergenic region was carried out by PCR using the Hybaid PCR Sprint temperature cycling system. The thermal profile involved an initial denaturing step at 94°C for 60 s, followed by 25 cycles of denaturing at 94°C for 30 s, annealing at 46°C for 30 s and elongation at 72°C for 90 s. An additional elongation was carried out for 120s at 72°C and a cooling step at 4°C for 60 s completed the reaction. Amplification fragments ranged from 3kb to approximately 500bp depending on the strain being amplified.

3.2.5 Southern Blot Hybridization.

Southern blot hybridization was carried out using the Dig detection system (Promega) in order to determine the number of 16S rRNA gene copies present within the different strains (ribotyping). Approximately 5μg off genomic DNA from the strains were digested with either *ScaI* or *BamHI* and the resulting restriction fragments separated on a

0,8% agarose gel. Neither of these enzymes has been shown to cut within the 16S rRNA gene. DNA was denatured in 0.25 M HCl, neutralised in 0.4 NaOH and transferred to a Hybond N^+ (Amersham) nylon membrane by means of overnight capillary blotting. The amplified 16S rRNA gene from two randomly chosen strains (Riv-14 and MT13) was 3'-end labeled with digoxigenin using the DIG oligonucleotide labelling and detection kit (Roche Biochemicals) and used as a hybridisation probe. Hybiridisation was done at 40°C followed by stringency washes at room temperature and 65°C. Membrane detection was preformed as per manufacturer's instructions (Roche Biochemicals).

3.2.6 Pulse Field Gel Electrophoresis (PFGE)

PFGE was done using a contour clamped homogeneous electric field (CHEF) system. Electrophoresis was done at 250V for 16h with a 20 second pulse time.

Intact genomic DNA was prepared by resuspending cell cultures in SET buffer to obtain an approximate OD600nm of 1.6. The cells were incubated at 37°C for one hour in the presence of 20mg/ml proteinase K (Merck). An equal volume of 1% low melting point agarose (Seaplaque) was added to the cell suspension. The cell suspension and agarose mix was applied to a 2x2x25-mm mold in order to create "plugs" containing embedded cells. Plugs were extracted from the mold and incubated in TE buffer containing 1% (w/v) SDS until plugs cleared. The embedded cells were lysed by incubating the plugs in ESP solution (Na-lauroyl sarcosine 10g/l, 168 g/l EDTA, pH 8, 1 mg/ml proteinase K) first for 30 minutes at 37°C and then overnight at 50°C for two days. The excess proteinase K was removed by washing the plugs in ES solution (ESP solution without proteinase K) overnight at 50°C. A further two 30 minute washes in TE buffer ensure the inactivation of any residual proteinase K. A proteinase inhibitor, Pefabloc (Boeringer Mannheim) was added to TE buffer and plugs were incubated overnight at 4°C. Plugs were stored in TE_{50} at 4°C until used. A 1% (w/v) agarose gel was prepared with 0.5x TBE buffer

3.3 Results and discussion

3.3.1 Number of 16S rRNA genes per genome

The genomic DNA from the thirteen *Sulfobacillus* strains and two *Alicyclobacillus* strains were subjected to restriction digestion. Restriction mapping from the 16S rRNA sequence was done in order to identify enzymes that does not have a sight within the 16S rRNA gene. Two enzymes namely *ScaI* and *BamHI* were shown not to have restriction sites within the 16S rRNA gene and were used in the restriction digestion of the gDNA. The digested restriction fragments were separated electrophoretically and analysed by means of Southern hybridization. 16S rRNA PCR products from strains MT13 and Riv-14 were labelled and used as hybridization probes. Because the enzymes used do not have an internal restriction site each band produced represents a single *rrn* operon. Hibridization results of gDNA digested with *BamHI* is shown in Figure 3.4. The results were confirmed by digesting the gDNA with *ScaI* and subjecting it to Southern hibridization (results not shown). Two distinct groups are visible, one which has three copies of the rrn operon and another with four rrn copies. Where the band numbers was difficult to determine, additional experiments were performed (data not shown).

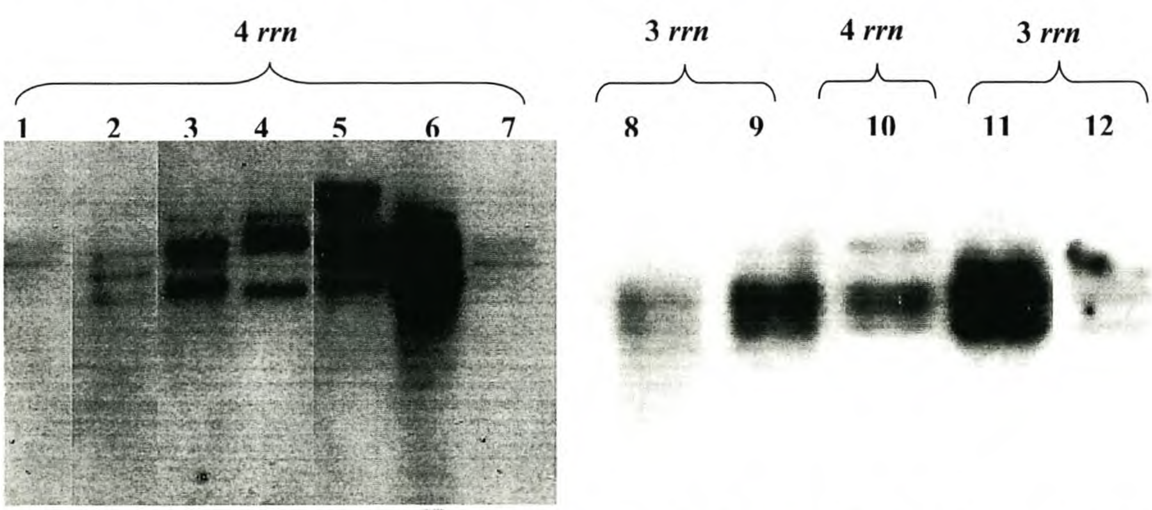

Figure 3.4 Ribotyping results obtained by Southern hybridization indicating the number of ribosomal rRNA operons per Sulfobacilli isolates. Genomic DNA was digested with *Bam*HI and transferred to a membrahne by means of capillary blotting. Results are of two different blots, band sizes are not comparable but banding patterns are. Lanes: **1**, Kara, **2**, THWX, **3**, L15, **4**, Riv-14, **5**, MT13, **6**, Adapt, 7, GG6/3, **8** GG6/2, **9**, TH1, **10**, 611, **11** ALV, **12**, YTF1

A direct determination of the number of 16S rRNA copies per strain was obtained by means of 16S-23S rRNA intergenic region PCR amplification (Fig.3.5). It has been noted by Jensen *et al* (1993) that primary and secondary amplification products are produced during intergenic region amplification. According to Jensen *et al* (1993) primary bands can be classifies as those bands of high reproducibility and intensity. Secondary bands are weaker and more variable. By manipulating the PCR condition it is possible to eliminate most if not all of the secondary products. Secondary bands that could not be eliminated by manipulation of the PCR conditions are used in conjunction with primary band as a means to differentiate between the different species.

All of the strains tested produced intense bands at 650bp and 750bp that can thus be classified as primary bands. Because of the inconsistency of the secondary fragments they are not very useful in the identification of species but may aid in confirmation of an already made identification. A comparison of the amblifiactions band sizes can be seen in Table 3.1.

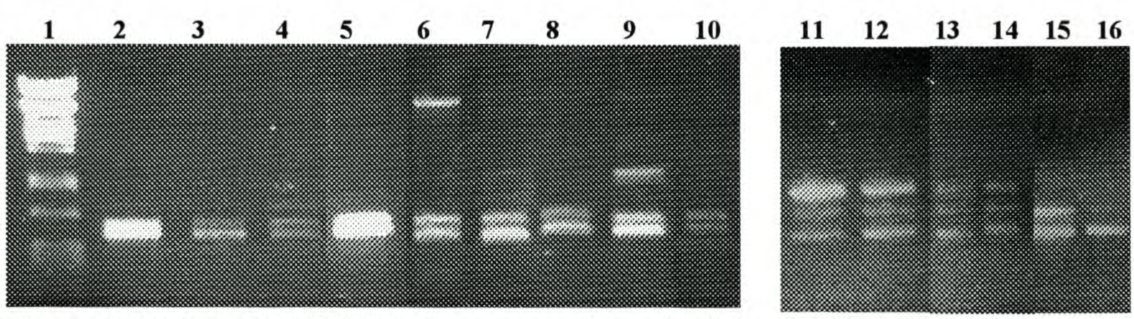

Figure 3.4 Number of the *rrn* genes as determined by PCR amplification of the intergenic region between the 16S rRNA and 23S rRNA genes.
Lanes: **1**, λ-*PstI*; **2**, TH1; **3**, GG6/2; **4**, GG6/3; **5**, ALV; **6**, THWX; 7, L15; **8**, SLC66; **9**, Riv-14; **10**, GSM; **11**, MT13; 12, Kara; **13**, Adapt; **14**, 611; **15**, YTF1; **16**, YTF5.
Band sizes between blots are not to scale, but the banding patterns are comparable

The group with four copies of the *rrn* operon can be divided further into three subgroups. The first of these groups (MT13, Kara, Adapt and 611) had three primary fragments of identical fragment sizes. These four isolates also form a tight 16S rRNA sequence based cluster (see Figure 3.6). The second subgroup consisting of strains L15 and Riv-14 (also phylogenetically very closely related) and the third subgroup consisting of strains GG6/3 and THWX with very dissimilar sizes of their third bands. The 1.2 kb bands present with strains L15 and Riv-14 are clearly secondary bans as their yield was markedly lower than that of the primary bands. Even though strain GG6/3 has a band of 950bp, it cannot be grouped within the first subgroup, as this band is clearly not a primary band as its yield was lower than that of the other two fragments. The secondary band of strain THWX is approximately 3kb in length and is much larger in size than any of the other bands. The intensity (yield) of this band is comparable to the two primary bands and therefore can be seen as a third primary band.

Tabel 3.1 Intergenic spacer region PCR amplification sizes

Isolate 4 *rrn* subgroup	Fragments (kb)	Isolate 3 *rrn* subgroup	Fragments (kb)
MT13	0.65; 0.75; 0.95	YTF1	0.65; 0.75
Kara	0.65; 0.75; 0.95	YTF5	0.65; 0.75
Adapt	0.65; 0.75; 0.95	GG6/2	0.65; 0.75
611	0.65; 0.75; 0.95	TH1	0.65; 0.75
		ALV	0.65; 0.75
L15	0.65; 0.75; 1,2	GSM	0.65; 0.75
Riv-14	0.65; 0.75; 1,2	SLC66	0.65; 0.75
GG6/3	0.65; 0.75; 0.95		
THWX	0.65; 0.75; 3.0		

3.3.2 16S rRNA sequence analysis

Of the fifteen strains used within this study, the 16S rRNA gene sequence was already available for eight of the strains (NCBI database). The full sequence of the remaining seven strains (611, Adapt, GG6/2, GG6/3, MT13, TH1 and THWX) was determined as part of this study by sequencing directly from the 16S rRNA PCR products. Sequencing was done in both directions.

Complete sequence alignments were obtained by making use of the full optimal alignment function of DNAMAN version 4.13.

A dendogram (Table 3.2) and a phylogenetic tree (Fig 3.6) were constructed from the aligned sequence data using the options provided by DNAMAN version 4.13.

The clustering of the strains correlated with the number of rRNA operons present within the strains. Isolates from the 3 *rrn Sulfobacillus* group had 16S rRNA sequence homology between 90.9% and 98.5% and formed a fairly tight phylogenetic cluster (Figure 3.6). Within the 4 *rrn Sulfobacillus* group, six strains (MT13, Kara, Adapt, 611, L15 and Riv-14) had high sequence identity to each other (94.1% - 99.4%, Table 3.2) and were also closely clustered on the phylogenetic tree (fig. 3.6). Based on the 16S rRNA sequence data, the two remaining 4 *rrn Sulfobacillus* isolates (GG6/3 and THWX) fell between these closely clustred groups. Although fairly closely related to each other (94.6% identity) isolates GG6/3 and THWX were approximately equally related tot he 3 *rrn* and 4 *rrn* copy Sulfobacilli.

In spite of having three copies of rrn genes with 16S-23S spacer region similar to the 3 rrn Sulfobacillus isolates, the two Alicyclobacillus isolates clearly formed a cluster of their own (Table 3.2, Fig. 3.6).

16S rRNA sequenc identity of at least 97% have been suggested as a guide to classify organisms as the same species (Chapter 1). Sequence identity of about 80% between most members of the 3 rrn and 4 rrn groups indicate that these two groups might even belong to two distinct genera. As the resolving power of the 16S rRNA sequence data is not very high, further intensive analysis such as DNA-DNA hybridization will have to be undertaken.

Table 3.2 Dendogram showing homology values as derived from the 16S rRNA gene sequences

	%Homology with:														
Isolate	Kara	MT13	Adapt	611	L15	Riv-14	GG6/3	THWX	GG6/2	YTF1	TH1	YTF5	ALV	GSM	SLC66
Kara	100%														
MT13	99.0%	100.0%													
Adapt	99.4%	99.3%	100.0%												
611	97.7%	97.1%	97.9%	100.0%				**4 *rrn***							
L15	95.0%	94.1%	94.8%	96.1%	100.0%										
Riv-14	94.9%	94.2%	94.6%	95.8%	100.0%										
GG6/3	87.7%	87.4%	88.3%	88.7%	89.2%	88.7%	100.0%								
THWX	90.0%	89.3%	90.3%	87.9%	87.6%	87.5%	94.6%	100.0%							
GG6/2	81.9%	82.2%	83.1%	81.8%	81.5%	81.2%	87.3%	92.5%	100.0%						
YTF1	81.6%	81.9%	82.8%	81.0%	80.5%	80.3%	87.0%	92.2%	97.6%	100.0%					
TH1	80.9%	87.1%	82.1%	80.6%	80.1%	80.0%	86.6%	91.8%	98.5%	97.9%	100.0%			**3 *rrn***	
YTF5	79.2%	75.4%	79.8%	79.6%	79.7%	79.5%	89.3%	92.6%	93.7%	93.5%	92.9%	100.0%			
ALV	79.6%	79.9%	80.8%	79.1%	78.7%	78.5%	85.3%	90.5%	96.6%	96.3%	96.9%	90.9%	100.0%		
GSM	68.7%	68.9%	69.8%	68.9%	68.9%	68.2%	66.9%	68.2%	70.8%	72.3%	71.3%	71.8%	71.5%	100.0%	
SLC66	69.0%	69.0%	70.0%	69.4%	69.5%	68.9%	67.4%	68.8%	72.6%	73.8%	72.4%	72.3%	72.6%	88.6%	100.0%

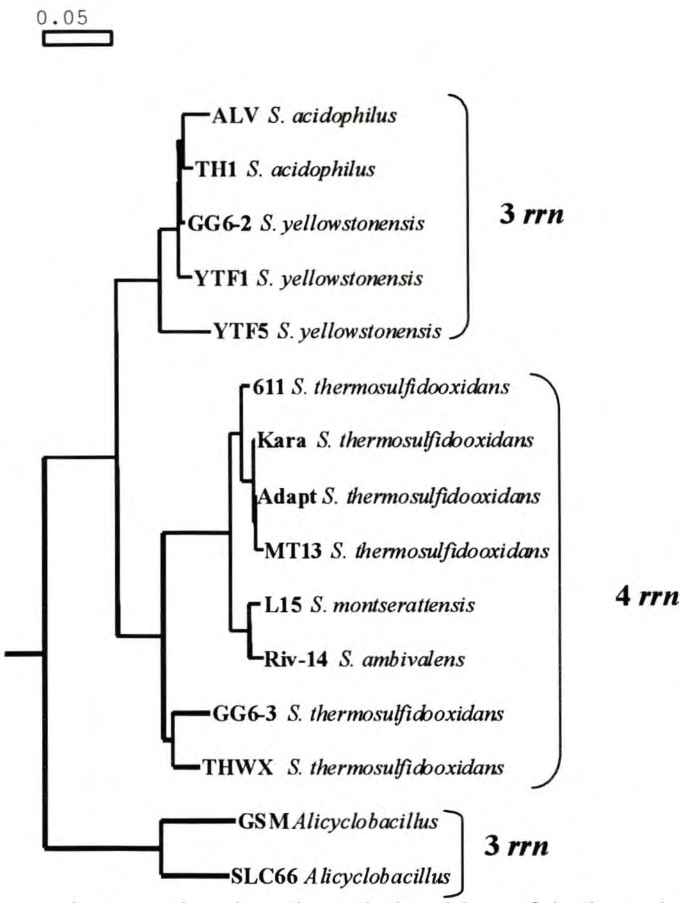

Figure 3.6 Phylogenetic tree showing the relationships of isolates belonging to the genera *Sulfobacillus* and *Alicyclobacillus*. The tree was constructed from the nearly full-length sequences of the 16S rRNA gene. Bootstrap values of 100 was used during the tree construction. The scale bar represents 0.5 estimated nucleotide change per position. The main two groups are indicated with brackets. Genbank accession numbers for those strains available are given in Table 2.1.

From the alignment data is was possible to examine the variable regions as they are portrayed by Brosius *et al.* (1987 and 1981) Conserved regions within variable region two (spanning from bp170 to bp 220, *E .coli* numbering) can be used to distinguish between the two main *rrn* groups (Fig.3.7). It is further possible to distinguish between the subgroups within the 4 *rrn* main group when comparing the sequence of variable region two. The tight phylogenetic cluster of MT13, Kara and Adapt (Fig.3.6) is illustrated by an examination of this region. It is interesting to note that strain THWX has sequence identical to the above mentioned strains within this region, even though it does not fall within the same phylogenetic cluster. Sequences from the two *Alicyclobacillus* strains show very little sequence similarity to any of the Sulfobacilli within any of the variable regions.

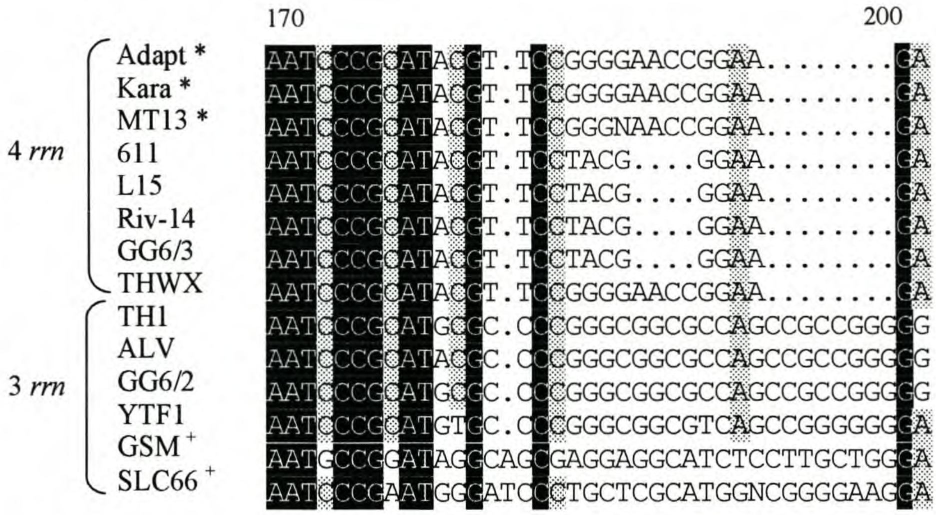

Figure 3.7 Multiple alignments of variable region two (E. coli nucleotide position 170 – 220bp). Separation of the two main groups are indicated with brackets. Strains belonging to the subgroup consisting of a tight phylogenetic cluster are indicated with an asterix (*). Note sequence similarities of strain THWX to the above-mentioned subgroup. Alicyclobacilli strains indicated with a plus (+) sign.

3.3.3 16S rRNA restriction enzyme mapping

Restriction enzyme mapping of amplified DNA is a simple and fast technique that can be used for strain or species identification. By comparing the 16S rRNA sequence of the strains studied several restriction enzyme sites were identified that would be helpful with the identification of isolates from the two major groups of Sulfobacilli. The presence rather than the absence of a restriction enzyem site has more value for identification purposes. For this reason nine enzymes (*Age*I, *Bsi*I, *Eco*RI, *Ehe*I, *Kpn*I, *Sac*I, *Sac*II, *Sal*I) were chosen that are capable of distinguishing between the two Sulfobacilli groups as well as Alicyclobacillus (Fig. 3.7). Even though several of the restriction enzymes are present within all groups, the position of their restriction sites is frequently different, making them acceptable for use in group identification.

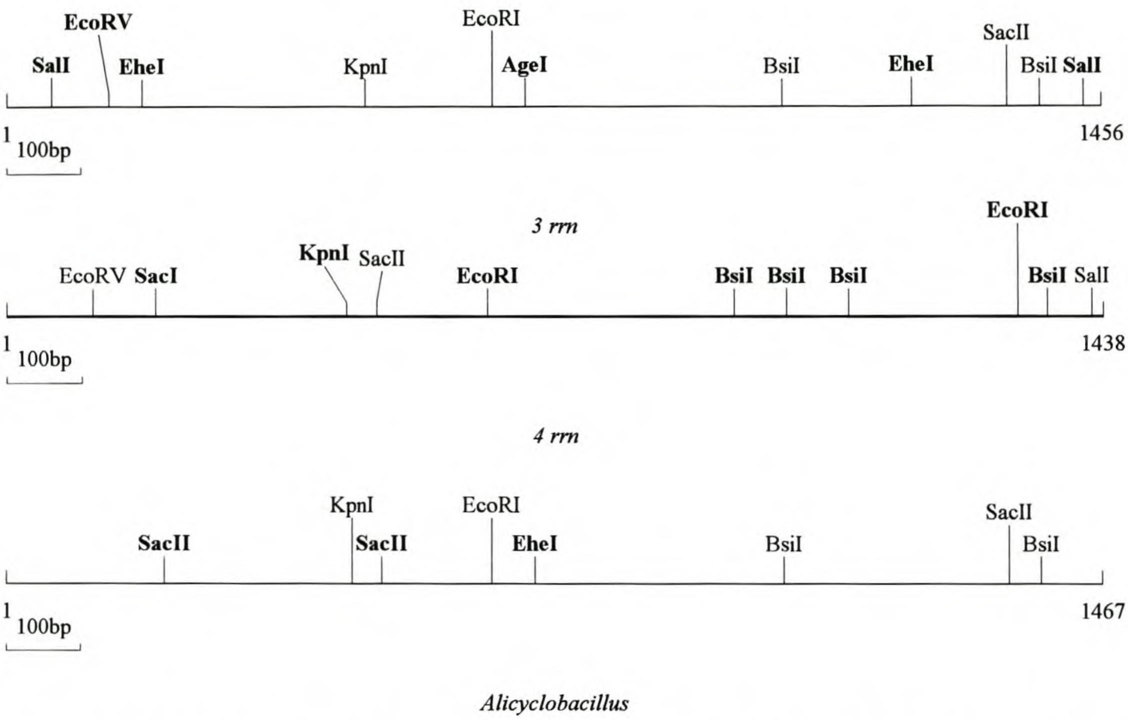

Figure 3.8 Map of nine restriction endonuclease sites within the 16S rRNA gene of both groups of *Sulfobacillus* (3 *rrn* and 4 *rrn*) and *Alicyclobacillus*. Restriction enzymes that can be used in order to distinguish a specific group have been placed in bold lettering.

As may be seen (Fig. 3.8) there are at least two restriction enzymes that could be used to distinguish between the three groups of organisms based on the isolates examined in this study.

3.3.4 Plasmid identification

Pulse field gel electrophoresis is routinely used in order to separate intact genomic DNA from smaller plasmid DNA. It was of great interest to identify Sulfobacilli, which possibly could contain one or more plasmids. These plasmids could in future be used as a genetic tool for introducing novel features such as metal resistance to the biomining organisms. Agarose plugs for all the Sulfobacilli isolates were prepared and the DNA separated overnight. Two of the isolates namely THWX and 611 showed bands that appeared to be plasmid bands (Fig 3.9). Degradation of DNA occureed resulting in smears across the lanes. The plasmids cannot be sized from these results seeing ass uncut DNA was run. P.R. Norris (pers. comm) has confirmed the presence of a plasmid within strain THWX.

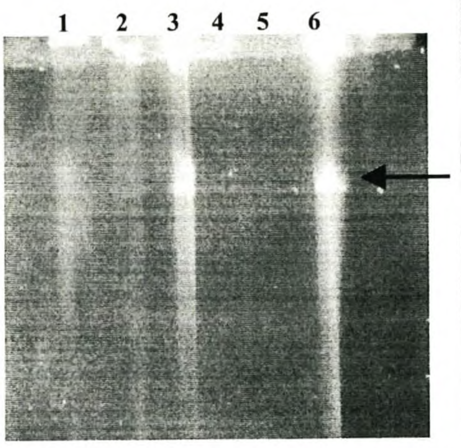

Figure 3.9. A PFGE indicating possible plasmids for two of the strains of Sulfobacilli. Dna degradation can be seen as smears down the lanes. The arrow indicates the plasmid band. Lanes: **1**, Riv-14, **2**, ALV, **3**, THWX, **4**, GG6/3, **5**, Kara, **6**, 611

Strain 611 is being investigated as part of a new project and the presence of two plasmids within this strain has been confirmed. One of these plasmids has already been isolated in the laboratory as part of a B.Sc Honours project (T.M. Joubert).

CHAPTER FOUR

GENERAL DISCUSSION

A large number of bacteria have been isolated that are capable of oxidizing minerals containing elemental sulfur (S^0), sulfides and ferrous sulfate. These include *At. ferrooxidans*, *At. thiooxidans*, *At. caldus*, *L. ferrooxidans* and *Sulfobacillus* species. Of the above mentioned bacteria, *Sulfobacillus* is one of the least studied with relatively little known about it's physiological, metabolic and genetic makeup. One objective of this study was to accumulate more information regarding the biodiversity of this organism. To this effect thirteen isolates from different geographical locations were chosen as a sample group. Two strains from the closely related genus, *Alicyclobacillus* were also included to be used for comparison. A polyphasic approach was undertaken, using both genetic and phenotypic tools in order to gain more knowledge regarding the diversity of these organisms.

It is known that the phenotypic characteristics of bacteria are not sufficient for the delineation of species (Roselló-Mora and Amann, 2001). In the case of iron- and sulfur-oxidizing bacteria this is especially problematic. This is mostly because of the limited morphological and physiological features that many of these bacteria exhibit. A phenetic comparison where a large set of characteristics is compared is normally used in order to show the degree of similarity between the different organisms under investigation. In this study a classical phenetic analysis was undertaken where several morphological, physiological and biochemical features of *Sulfobacillus* and *Alicyclobacillus* were compared.

As was expected very few morphological differences were noted between the isolates of *Sulfobacillus*. Two strains namely ALV and GG6/3 gave Gram variable results. It can however be deduced that they are Gram positive because of their close phylogenetic association with the other *Sulfobacillus* isolates under investigation as well as the observation that both strains were able to produce endospores under energy poor conditions. Variations in results of Gram staining with strain ALV and several other strains belonging to *Sulfobacillus* and related bacteria have been noted by several other researchers (Marsh and Norris 1983; Goebel and Stackebrandt, 1994;Brierley J.A., 1997).

Two groups of *Sulfobacillus* with different optimal growth temperatures of 45°C and 55°C respectively have been identified. As neither of the two groups grew well at temperatures of 60°C and above, it can be postulated that *Sulfobacillus* species will flourish in biooxidation processes of between 45°C – 55°C. With respect to pH tolerance the differences between the Sulfobacilli was less marked and their pH optima (pH 2.5) and pH range (pH 1.5 – pH 5.0) were identical. The single time interval that was presented in the determination of the optimal growth pH and temperature was chosen to be as late as possible to allow for maximum growth for comparative analysis without there being to much precipitate or jarosite formation.

Two strains (Adapt and 611) were isolated from Billiton research plant (BRP) in South Africa and identified as *Sulfobacillus*-like bacteria (S.M. Deanne, University of Stellenbosch, perss. com). Strain Adapt was isolated from tanks operating at 55°C whereas strain 611 was isolated from tanks operating at 40°C. These strains were included in the study in order to determine which *Sulfobacillus* species were present within industrial biooxidation plants of South Africa. It is important to know which species flourish within the South African biooxidation plants in order to plan future research for the optimization of the biooxidation processes. Once it has been established which species are prevalent, investigations into the development of genetic systems specific for those species can be undertaken. This can lead to the improvement of the organisms (e.g. metal tolerance), which in turn will lead to an improvement in their bioleaching capabilities. It was surmised that because the isolates were collected from tanks operating at two different temperatures that they would likely belong to different species. Investigation into their phylogeny (16S rRNA sequence similarities and ribotype results) however has shown that both of the South African strains belong to the same species, namely *S. thermosulfidooxidans*. When pulse field gel electrophoresis was employed in order to determine if any of the strains under investigation possibly contained any plasmids, the South African strain 611 as well as strain THWX isolated from Wales gave positive results. The possibility of plasmids in strain 611 was an important find as it opens the doorway for future research into the development of a genetic transfer system for species found within South African biooxidation processes. As pointed out earlier, one of these plasmids has already been isolated (T.M. Joubert, pers. comm.). Initial restriction mapping of the plasmid has been completed and sequencing in order to determine what genes are carried on the plasmid is under way.

One of the most important criteria of this study was to develop a convenient system for the identification of *Sulfobacillus* species.

The *Sulfobacillus* species examined were clearly divided into two groups based on the analysis of their 16S rRNA gene sequences as well as the number of ribosomal (*rrn*) operons present. The first logical step in identifying the group to which an isolate belongs is to make use of restriction enzyme digestion of the 16S rRNA gene. This technique is simple, fast and effective. Figure 3.7 shows the restriction enzyme maps for the two *Sulfobacillus* groups. From this map, appropriate enzymes can be chosen that will give distinctive banding patterns depending on which group of *Sulfobacillus* the isolate belongs to.

Because of the relative ease of determining the 16S – 23S rRNA spacer regions within the organisms by means of PCR amplification, it can be used to confirm the grouping of the isolate as determined by the restriction enzyme digestion. The amplification products will place the isolate either within the 3 *rrn* group (two primary bands) or the 4 *rrn* group (two primary bands as well as another secondary band of variable size) (Table 3.1).

As the 4 *rrn* group produces two primary amplification products as well as a secondary product of variable size, the sizes of the secondary bands can be used in order to narrow down the species search. Secondary amplification product sizes are not normally used in species identification, as their presence is not always guaranteed. In this study however it was found that after PCR optimization, secondary bands were always present and could therefore be used to aid species identification.

Strains belonging to *S. thermosulfidooxidans* have a secondary band of approximately 950bp that can be used in the species identification. It is more difficult to place an isolate within the other species belonging to the 4 *rrn* group as there seem to be more than one possibility of secondary band size for *S. ambivalens*. Another factor that makes the placing difficult is the fact that strain Riv-14 classified as a *S. ambivalens* and strain L15 considered to be a *S. montserattensis* have similar secondary band sizes. It is likely that these two isolates are the same species. The same problem occurs with *S. yellowstonensis* and *S. acidophilus* within the 3 *rrn* group as only the two primary bands are present for both of the species. It is interesting to note that strain Riv-14 classified as a *S. ambivalens* and strains THWX and GG6/3 also belonging to this species, gives very different 16S –23S spacer region banding pattern. When taking the large phylogenetic (evolutionary) distance that separate the latter two strains from strain Riv-14, it can be surmised that these three strains do not belong to the same species (Fig.3.5 and Fig. 3.6). Again it would appear that Riv-14 might not be an *S. ambivalens*. Even though strains placed within the species *S. yellowstonensis* and *S. acidophilus* also gives the same 16S – 23S spacer region banding patterns, their phylogenetic (evolutionary) distance is far smaller than that of the strains of *S. ambivalens*. According to this study strain Riv-14 is more closely related to strain L15 belonging to *S. montserattensis* (Table 3.2) and should therefore be considered as also belonging to species *S. montserattensis*. Although these two species exhibit different sugar utilization capabilities, it is important to remember that genomic information is more stable in species identification than phenotypic information. The major reason for this is that phenotypic traits give an indication of only a very small part of the complete genomic information content of an organism.

Once the preliminary species identification has been established the utilization of different carbon sources by the different *Sulfobacillus* species can be employed to aid further distinction. The carbon source utilization of the species is sufficiently different in order to aid the species identification (Table 2.4). Strains belonging to the 3 *rrn* group can be divided into the two different species by comparing their ability to utilize glucose, arabinose and xylose under mixotrophic conditions. All of the strains belonging to *S. acidophilus* had the ability to utilize these sugars whereas none of the strains belonging to *S. yellowstonensis* were able to utilize these sugars under mixotrophic conditions. By determining the ability of the isolate from the 4 *rrn* group to use galactose, arabinose and xylose a distinction between *S. montserattensis* and *S. ambivalens* can be drawn with the latter being unable to utilize these sugars during mixotrophic growth.

If at this point the species to which the isolate under investigation belongs is unclear, sequencing of the complete 16S rRNA gene can be undertaken. This sequence can be alignmened to known 16S rRNA sequences belonging to the two different groups of Sulfobacilli. This technique however is the most labor intensive and expensive of all the techniques described thus far.

Sulfobacillus can be regarded as one of the least metabolically restricted biomining bacteria known because of it's ability to utilize more than one energy source. This study has also indicated that most strains of the *Sulfobacillus* have the ability to respire anaerobically making use of ferric iron (Fe^{3+}) as an electron donor.

From phylogenetic data obtained from the comparison of the 16S rRNA gene sequences as well as the number of 16S *rrn* operons present it is clear that *Sulfobacillus* can be divided into at least two subgroups. The 16S rRNA sequence similarities between the two groups are fairly low, which could indicate that what we thought of as one genus (*Sulfobacillus*) may actually be two separate genera (Fig. 3.5 and 3.6).

It has been stated previously that the use of 16S rRNA gene sequence alone for the delineation of the species is not acceptable because of the highly conserved nature of this gene between closely related organisms (Chapter one). The use of the 16S rRNA gene sequences however is generally robust for the delineation of taxa above that of the species level (Roselló-Mora and Amann, 2001). It has been suggested that no two organisms can belong to the same species if their overall mol% G+C ratio differs by more that 5% (Wayne *et al.*, 1987). Previously published data regarding the mol% G+C ratios of the two species *S. thermosulfidooxidans* and *S. acidophilus* has reported that their mol% G+C ratios are 48-50% and 55-57% respectively (Norris *et al.*, 1996). These values fall on the borderline of the recommended 5% difference thus indicating that they may be two different but related genera. No concise conclusions can be drawn however as to whether more than one genus is represented without the degree of overall DNA-DNA similarities being known.

Information regarding the diversity within the genus *Sulfobacillus* as obtained from this study is by no means a complete representation of the variation found within Sulfobacilli. The results obtained however have contributed to the development of an easy to follow identification system for *Sulfobacillus* species. Furthermore, the identification of the species of *Sulfobacillus* found within the South African biooxidation plants has opened a door for future research into the optimization of biooxidation processes running at temperatures between 45°C – 50°C. Identifying South African strain 611 as a plasmid containing strain has opened the field for the development of a genetic transfer system that can further improve the biooxidation processes.

With the development of a convenient, fast and cost-effective identification system for the *Sulfobacillus* species, the identification of *S. thermosulfidooxidans* as the species present within South African biooxidation plants as well as filling in some of the gaps in the knowledge of the biodiversity of *Sulfobacillus* and the related organism, *Alicyclobacillus,* all of the aims as set out in the beginning of this study have been successfully completed.

Appendix A

Media, buffers and solutions

All media, buffers and solutions were sterilized by autoclaving at 121°C for 20 minutes. Heat liable substances were filter sterilized using a 0.22 µm membrane filter (Millipore)

A1 Liquid Media

A1.1 10x basal salt solution

$(NH_4)_2SO_4$	12.5 $g.l^{-1}$
$MgSO_4.7H_2O$	5.0 $g.l^{-1}$

pH to 2.5 with H_2SO_4 (autoclave)

A1.2 0.5 M $FeSO_4$

$FeSO_4.7H_20$	139.0 $g.l^{-1}$

PH < 1.3 with H_2SO_4 (autoclave)

A1.3 25 mM Ferric sulphate

Ferric sulphate	10.0 $g.l^{-1}$

A1.4 1 M Glycerol

Glycerol	73.0 ml/l^{-1}

A1.5 1000x trace elements

$ZnSO_4.7H_2O$	10.0 $g.l^{-1}$
$CuSO_4.5H_2O$	1.0 $g.l^{-1}$
$MnSO_4.4H_20$	1.0 $g.l^{-1}$
$CoCl_2.6H_2O$	0.5 $g.l^{-1}$
$Cr_2(SO_4)_3.15H_2)$	0.5 $g.l^{-1}$
$Na_2B_4O_7.10H_2O$	0.5 $g.l^{-1}$
$NaMoO_4.2H_2O$	0.5 $g.l^{-1}$

Add 530 µl H_2SO_4 (autoclave)

A1.6 0.5M Potassium tetrathionate

$K_2S_4O_6$	151.3 $g.l^{-1}$

Filter sterilize

A1.7 Ferrous sulphate media

Basal salts, trace elements, iron solution and potassium tetrathionate were mixed aseptically and the pH adjusted to pH1.8 with H_2SO_4.

A1.8 Yeast extract (YE) media

Basal salts and yeast extract were combined to final concentration, the pH adjusted to pH2,5 with H_2SO_4 and autoclaved. Filter sterilized iron solution is added aseptically.

A2 Solid Media

A2.1 Ferric iron solid media

Add ferric sulphate, glycerol, basal salts and yeast extract to final concentration. Adjust pH to pH1.8 with H_2SO_4 and autoclave. Simultaneously make up 1.2 % agarose and autoclave. Let both mixtures cool to approximately 50°C and mix. Pour plates and let set.

A2.2 SJH Overlay-plates

A: Add 0.25 triptone soya mix ,$(NH_4)_2SO_4$ 1.0 and $MgSO_4$ 0.4 (w/v) together, adjust the pH to pH 2.5 with H_2SO_4 and autoclave.

B: Prepare 1.0% agarose and autoclave

Prepare two bottles of each.

Add 0.5 M ferrous sulphate (1 ml/20 ml), 1000x trace elements (1 µl/5ml) and potassium tetrathionate (1 ml/100 ml) to both bottles of mix A.

Mix one bottle of mix A and mix B together and pour (just covering the bottom).

Leave to set.

To mix A of remaining bottles, add 10ml actively growing SJH (adapted to potassium tetrathionate).

Mix bottles A and B together and pour on top of bottom layer.

A3. Buffers and solutions

A3.1 EDTA (Ethylene diamine tetracetic acid, 0.5 M pH8)

EDTA.2H_2O	168.1 g

Distilled H_2O to 1000 ml

pH adjusted with NaOH (10 N) to pH 8.0

A3.2 20x SSC

NaCl	175.3 $g.l^{-1}$
Sodium citrate	88.2 $g.l^{-1}$

pH adjusted with NaOH (10 N) to pH 7.4; autoclave

A3.3 TE buffer

Tris	1.21 $g.l^{-1}$
EDTA	0.34 $g.l^{-1}$

pH adjusted to pH 8.0 (HCL); autoclave

A3.4 10x TBE

Tris	108.0 $g.l^{-1}$
Boric acid	55.0 $g.l^{-1}$
EDTA	40.0 $ml.l^{-1}$

Distilled H_2O to 1000ml

A3.5 ES solution

EDTA	16.8 g

Na-lauroyl sarcosine 1.0 g

Distilled H_2O to 100ml

pH adjusted to pH 8.0 with NaOH (10N); autoclave

A3.6 ESP solution

ES solution containing 1 mg/ml proteinase K

A3.7 TE_{50} for PFGE

Tris	3.6 $g.l^{-1}$
EDTA	16.8 $g.l^{-1}$

pH adjusted to pH 7.6 (HCL); autoclave

A3.7 50 mM stock Pefabloc SC (Roche Biochemicals)

2 mg/167 µl distilled H_2O

Store at –20°C

Use at a final concentration of 1-5 mM

A3.8 Proteinase K

10 mg/ 500 µl distilled H_2O

A3.9 0.4 N NaOH

NaOH 16 $g.l^{-1}$

A3.10 0.25 M HCL

HCl (conc) 21.34 $ml.l^{-1}$

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